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CARNE DE VACUNO ENRIQUECIDA CON *n-3* Y CLA Y ACTITUD DE LOS CONSUMIDORES FRENTE A ALIMENTOS FUNCIONALES

APTITUD TECNOLÓGICA PARA LA ELABORACIÓN DE NUEVOS PRODUCTOS SALUDABLES

TESIS DOCTORAL

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Carne de vacuno enriquecida con *n-3* y CLA y actitud de los consumidores frente a alimentos funcionales. Aptitud tecnológica para la elaboración de nuevos productos saludables.

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Los resultados obtenidos en esta tesis han dado lugar a los siguientes artículos científicos y comunicaciones en congresos:

Artículos

- Autores: Albertí, P., **Gómez, I.**, Mendizabal, J.A., Ripoll, G., Barahona, M., Sarriés, V., Insausti, K., Beriain, M.J., Purroy, A., Realini, C.
  Título: Effect of whole linseed and rumen-protected conjugated linoleic acid enriched diets on feedlot performance, carcass characteristics, and adipose tissue development in young Holstein bulls.
  Ref. revista: Meat Science
  Volumen: 94 Páginas, inicial: 208 final: 214 Fecha: 2013
- Autores: Realini, C.E., Kallas, Z., Pérez-Juan, M., **Gómez, I.**, Olleta, J.L., Beriain, M.J., Albertí, P., Sañudo, C.
  Título: Relative importance of cues underlying Spanish consumers' beef choice and segmentation, and consumer liking of beef enriched with n-3 and CLA fatty acids.
  Ref. revista: Food Quality and Preference
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- Autores: **Gómez, I.**, Mendizabal, J.A., Sarriés, M.V., Insausti, K., Albertí, P., Realini, C., Pérez-Juan, M., Oliver, M.A., Purroy, A., Beriain, M.J.
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- Autores: **Gómez, I.**, Beriain, M.J., Mendizabal, J.A., Realini, C., Purroy, A.
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- Autores: **Gómez, I.**, Beriain, M.J., Mendizabal, J.A.
  Título: Shelf life of low-fat beef patties enriched with omega-3, CLA and oleic fatty acids and influence of grape seed extract to inhibit lipid oxidation.
  (en elaboración)

Comunicaciones

- Autores: Sarriés, M.V., Mendizábal, J.A., Beriain, M.J., Insausti, K., **Gómez, I.**, Sanz, M., Albertí, P., Purroy, A.
  Título: Efecto de la suplementación con dietas enriquecidas en ácidos grasos n-3 y en CLA en el perfil de ácidos grasos conjugados de la carne de terneros frisones.

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- Autores: **Gómez, I.**, Insausti, K., Marín, R., Mendizábal, J.A., García, S., Sarriés, M.V., Zudaire, G., Beriain, M. J.

  Título: Effect of grape seed extract on colour, sensory properties and oxidative stability of beef.

  Tipo de participación: Póster

  Congreso: 57th ICoMST

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  Lugar de celebración: Ghent, Bélgica Fecha: 2011

- Autores: **Gómez, I.**, Mendizabal, J.A., Beriain, M.J., Albertí, P., Sarries, M.V., Arana, A., Insausti, K., Soret, B., Purroy, A.

  Título: Effect of supplementation with linseed and CLA on adipose tissue cellularity of Holstein young bulls.

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  Congreso: 63rd Annual Meeting of the EAAP – European Federation of Animal Science.

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- Autores: **Gómez, I.**

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  Lugar de celebración: Oviedo, España Fecha: 2012

- Autores: **Gómez, I.,** Beriain, M.J., Mendizábal, J.A., Sarriés, M.V., Pau, V. Insausti, K., Albertí, P., Realini, C.E., Purroy, A.

  Título: Efecto de dietas enriquecidas con semilla de lino y CLA en el perfil de ácidos grasos del depósito subcutáneo de terneros frisones.

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- Autores: Gómez, I., Mendizabal, J.A., Sarriés, M.V., Insausti, K., Albertí, P., Realini, C., Purroy, A., Beriain, M.J.
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  Tipo de participación: Póster
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Resumen

La innovación es un factor clave para impulsar y consolidar el sector cárnico, incrementando la competitividad en la cadena de carne de vacuno y garantizando a los consumidores los mejores productos, sanos y de calidad.

El objetivo general planteado en la presente Tesis Doctoral ha sido estudiar el efecto de la incorporación de ingredientes ricos en ácidos grasos poliinsaturados *n-3* y CLA en las dietas de cebo de terneros con objeto de mejorar la calidad nutricional y organoléptica de su carne en función de las preferencias de los consumidores nacionales y posibilitar la obtención de nuevos derivados cárnicos saludables.

Se utilizaron 48 machos enteros Holstein de 150 kg de peso vivo que fueron alimentados con 4 dietas experimentales (12 animales por tratamiento):

1. CONTROL: pienso concentrado comercial enriquecido.
2. LINO: CONTROL con un 10% de lino.
3. CLA: CONTROL con un 2% de CLA.
4. LINO+CLA: CONTROL con un 10% de semilla de lino y 2% de CLA.

El sacrificio se realizó cuando los animales alcanzaron 450 kg de peso vivo (10-11 meses de edad). A las 24 horas del sacrificio se procedió a la toma de muestras del músculo *Longissimus Dorsi*, que posteriormente fueron congeladas hasta la realización de los diferentes análisis y experimentos que se han llevado a lo largo de la tesis.

El enriquecimiento con un 10% de semilla de lino y 2% de CLA de las dietas, que no ha afectado a los parámetros productivos ni metabolismo del tejido adiposo, ha mejorado el perfil de ácidos grasos de la carne, adecuándose más a los requerimientos nutricionales de los consumidores. Asimismo, esta carne enriquecida ha mejorado su calidad organoléptica en función de las preferencias de los consumidores nacionales y ha presentado una buena aptitud tecnológica.

Finalmente, se puede concluir que la inclusión de semilla de lino y CLA en las dietas de terneros Hosltein puede mejorar la calidad nutricional y sensorial de la carne, sin afectar negativamente al metabolismo del animal ni a la aptitud tecnológica de las carnes obtenidas.

Summary

Innovation is key to promoting and consolidating factor in the meat sector, increasing competitiveness in the beef chain and guaranteeing consumers the best products, healthy and quality.

The overall objective of this Doctoral Thesis was to study the effect of incorporation of ingredients rich in polyunsaturated fatty acids n-3 and CLA in the diets of fattening calves to improve the nutritional and organoleptic quality of its beef depending on preferences of consumers and to enable the development of new healthy meat products.

Forty-eight Holstein bulls of 150 kg live weight were fed 4 experimental diets (12 animals per treatment) were used:

1. CONTROL: concentrated feed enriched commercial.
2. LINO: CONTROL with 10% of whole linseed.
3. CLA: CONTROL with 2% of CLA.
4. LINO+CLA: CONTROL with 10% of whole linseed and 2% of CLA.

The slaughter was performed when the animals reached 450 kg live weight (10 to 11 months old). Within 24 hours of slaughter proceeded to sampling *Longissimus Dorsi* muscle, which were subsequently frozen until the completion of the different analyzes and experiments that have been carried along the thesis.

The enrichment with 10% flaxseed and 2% CLA diets, which did not affect growth performance and adipose tissue metabolism, improved fatty acid profile of the meat, adapting more nutritional requirements consumer. Furthermore, this enriched meat organoleptic quality has improved according to the preferences of domestic consumers and presented a good technological aptitude.

Finally, it can be concluded that the inclusion of flaxseed and CLA in the diets of calves Hosltein can improve the nutritional and sensory quality of the meat, without adversely affecting the metabolism of the animal or the technological aptitude of the meat produced.

1. Revisión bibliográfica

1. Introducción

La carne de vacuno es percibida por muchos consumidores como una carne poco saludable con un elevado contenido de grasas saturadas (AGS). Los AGS se han asociado con el aumento del colesterol en sangre y del riesgo de enfermedades cardiovasculares (las cuales son la causa principal de mortalidad en países desarrollados). Un bajo cociente AGPI/AGS y una relación n-6/n-3 elevada en la carne contribuyen al desequilibrio en el consumo de AG. Se ha considerado que los bajos cocientes AGPI/AGS de las dietas occidentales constituyen el principal factor de riesgo para el desarrollo de enfermedades cardiovasculares (Ganji et al. 2003, Katan 2000). Las dietas occidentales presentan además cocientes n-6/n-3 elevados (15-17:1) que favorecen el desarrollo de enfermedades cardiovasculares, cáncer y enfermedades inflamatorias e inmunitarias (Simopoulos 2002). En la población adulta española (25-60 años) el índice de obesidad es del 14,5% mientras que el sobrepeso asciende al 38,5%. El Ministerio de Sanidad y Consumo español ha manifestado que la obesidad es uno de los mayores retos de la salud pública del siglo XXI, representando los costes asociados a esta enfermedad en el año 2005 el 7% del gasto sanitario total (2.500 millones de euros anuales).

La carne representa un 10-20% de las calorías totales en las dietas de países industrializados y está documentado que la carne de rumiantes tiene un contenido elevado en AGS de hasta el 50% (Chizzolini et al., 1999). Por lo tanto, la estrategia de modificar el perfil de AG de la carne durante su fase productiva ofrecería al consumidor un producto que se adecúa más a las recomendaciones nutricionales. El sistema de engorde de terneros más utilizado en España se basa en dietas con un contenido elevado de concentrado y muy limitado de forrajes, lo cual produce una carne con una composición de AG n-6 excesivamente elevada desde el punto de vista de la salud humana. Existe la posibilidad de enriquecer el contenido de la carne con AG n-3 y CLA a través, por ejemplo, de la suplementación con lino y CLA protegido. Sin embargo, el aumento de los AGPI y en particular de los n-3 en la carne pueden afectar su estabilidad oxidativa requiriendo el uso de antioxidantes como la vitamina E. Existen indicios además de que el CLA podría mejorar la estabilidad de los lípidos extendiendo la vida comercial de la carne y sus productos

aunque la información es muy limitada sobre su efecto en la calidad de la carne de vacuno.

Varios estudios han demostrado el potencial de enriquecer la carne de vacuno con AG n-3 (Wood et al. 2008, Mach et al. 2006) y con CLA (Gillis et al. 2004, Richardson et al. 2006).

Los AG n-3 y en particular el eicosapentaenoico (EPA, 20:5) y docosahexaenoico (DHA, 22:6) juegan un papel importante en la salud y el desarrollo humanos. Estos AG están involucrados en el desarrollo de los tejidos cerebral y retinal, y en el progreso y la prevención de enfermedades de corazón y algunos cánceres (Simopoulos 1999, Connor 2000). La carne, el pescado, los aceites de pescado y los huevos son las únicas fuentes significativas de AG n-3 de cadena larga en la dieta. La carne tiene concentraciones menores de estos AG comparada con el pescado. Sin embargo, dado que el consumo de pescado es bajo en las dietas de numerosos países y de un número importante de consumidores dentro de cada país, es importante elevar el contenido de estos AG en la carne de vacuno (Scollan et al. 2001).

El ácido linoleico conjugado (CLA) representa a una mezcla de isómeros geométricos y de posición del ácido linoleico (cis-9, cis-12, ácido octadecadienoico), con dobles enlaces conjugados. Se forma a través de la isomerización por bacterias del rumen del ácido linoleico (18:2 n-6) y a través de la desaturación en el tejido adiposo por la enzima Δ9-desaturasa de otro producto de la biohidrogenación, el ácido trans-vaccénico (t11 18:1, AV; Griinari y Bauman, 1999). Existe potencial para enriquecer el contenido de la carne con CLA y AV a través del aumento de la disponibilidad del sustrato en la dieta animal, el ácido linoleico.

Con una industria alimentaria competitiva y cada vez más enfocada al consumidor, es necesario evaluar la aceptabilidad sensorial de la carne enriquecida con AG n-3, CLA y vitamina E por parte de los consumidores, la actitud de los mismos en contraposición con los productos funcionales y su disposición a pagar más por un producto diferenciado. Existe información en porcino que indica que la carne enriquecida con CLA provoca una reducción en la percepción de los consumidores del sabor, jugosidad y terneza y una disminución de la terneza

instrumental del producto (Dunshea et al. 2005). Pero no hay información disponible en vacuno sobre el efecto del enriquecimiento de una carne con CLA sobre la percepción sensorial de los consumidores.

La industria cárnica está constantemente trabajando para satisfacer las expectativas del consumidor y obtener una elevada calidad de productos a un coste razonable. El consumidor busca en los nuevos alimentos características tan variadas como placer, bienestar, seguridad y especialmente beneficios saludables. Por ello, se están desarrollando productos que presentan un elevado valor nutritivo como resultado de la reducción significativa del contenido en grasa, especialmente saturada, y aporte calórico, así como de la mejora del perfil lipídico adicionando otras fuentes como puede ser el aceite de oliva. El enriquecimiento en ácidos grasos insaturados de nuevos derivados cárnicos puede derivar en problemas de oxidación, por lo que el empleo de antioxidantes será necesario. El extracto de semilla de uva es un antioxidante natural que además de retardar los procesos de oxidación, presenta efectos beneficiosos en la salud de los consumidores.

La presente Tesis Doctoral, que se enmarca dentro de un proyecto nacional INIA (RTA2009-00004-CO2), plantea avanzar en el conocimiento del metabolismo y la deposición del tejido graso en vacuno, cuando se modifica el perfil de ácidos grasos de la carne, para lograr un producto que se adecúa más a los requerimientos nutricionales de los consumidores en condiciones de producción nacionales. Interesa especialmente estudiar la calidad tecnológica de la carne enriquecida con n-3, CLA, su vida comercial y su aceptabilidad sensorial. El desarrollo de una carne de mejor calidad nutricional y organoléptica en función de las preferencias de los consumidores nacionales, permitirá al sector del vacuno competir más eficientemente con otros sectores de la agroalimentación.

2. Incorporación de lípidos en la nutrición de los rumiantes

2.1. Metabolismo lipídico en los rumiantes

El alimento de los rumiantes, basado en forrajes ricos en fibra y compuestos almidonados, está formado por agua y materia orgánica, siendo los carbohidratos, proteínas y lípidos sus grandes grupos químicos moleculares, aunque estos nutrientes no pueden ser utilizados directamente por el animal. Para su adecuada digestión y asimilación los rumiantes poseen una cámara fermentativa pre-gástrica (retículo, rumen y omaso) y una cavidad gástrica (el abomaso) con mucosa secretora e idéntica funcionalidad a la de los estómagos de monogástricos. El alimento comienza su degradación con el proceso de rumia, que es la capacidad del rumiante para remasticar el alimento ingerido que se encuentre en el retículo-rumen mediante su regurgitación. Esta acción mecánica reduce el tamaño de las partículas pero no las hace disponibles para el animal todavía.

El metabolismo lipídico de los rumiantes (Figura 1) es muy distinto al de los monogástricos en diferentes aspectos que están relacionados con las modificaciones que los nutrientes de la dieta sufren por la fermentación microbiana ruminal (Bell, 1982). El rumen es un ecosistema complejo, compuesto por poblaciones de microorganismos anaerobios (10^{10}-10^{11} bacterias/ml, 10^{8}-10^{9} metanogenos/ml, 10^{6} hongos/ml y 10^{6} protozoos ciliados/ml de contenido ruminal) (Kumar *et al.*, 2009).

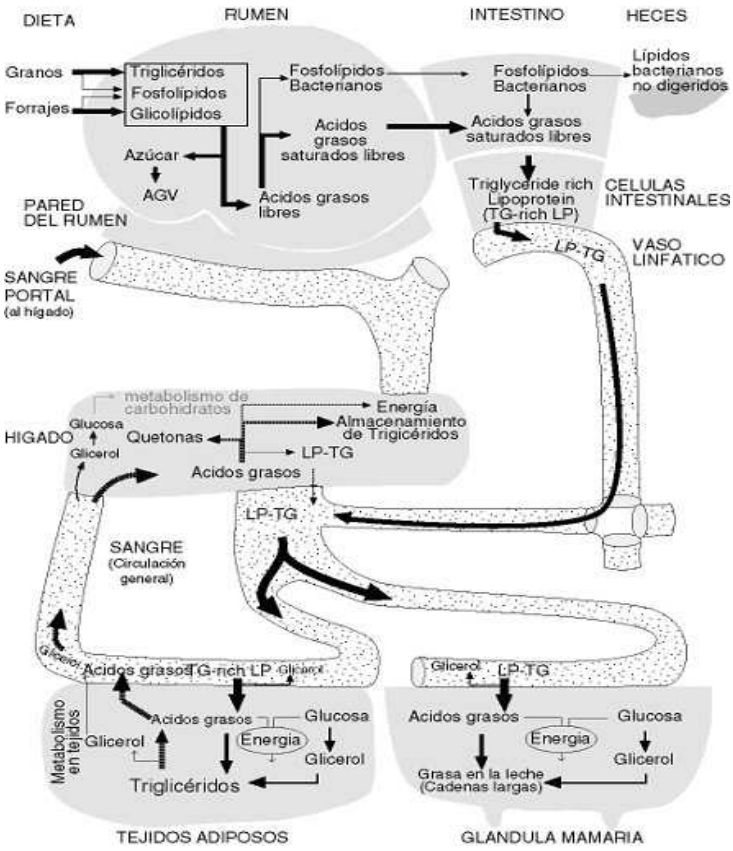


Figura 1. Metabolismo lipídico de los rumiantes (Wattiaux y Grummer, 2012).

En el rumen los lípidos de la dieta son sometidos a hidrólisis por lipasas microbianas, para que en un segundo paso los ácidos grasos insaturados libres sean biohidrogenados (Figura 1), siendo así los ácidos grasos absorbidos en el intestino más saturados que los presentes en la dieta (Doreau y Chilliard, 1997). Además, el metabolismo de las grasas en el rumen involucra otros procesos bioquímicos, tales como la síntesis de ácidos grasos microbianos y la absorción directa de los ácidos grasos menores de 14 átomos de carbono (Jenkins, 1993). Los ácidos grasos digeridos en el intestino delgado pueden tener diferentes destinos o usos metabólicos, siendo el principal el metabolismo energético en el animal, por lo que los lípidos son sintetizados y depositados (lipogénesis) o degradados (lipólisis) en respuesta al balance energético del animal.

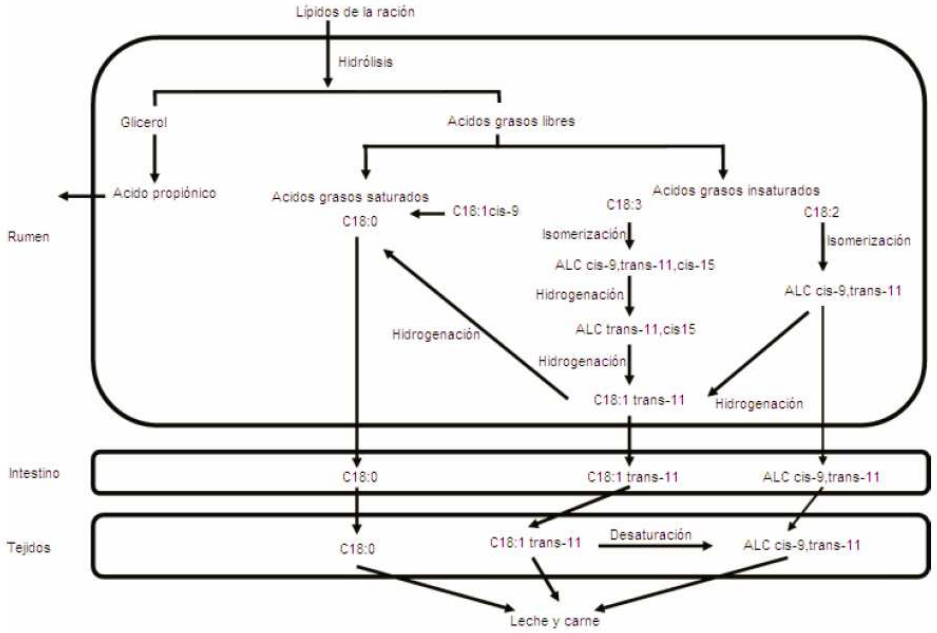


Figura 2. Digestión de los lípidos en el rumen. Adaptado de Tanaka (2005). Leyenda: C18:0, ácido esteárico; C18:1, ácido oleico; C18:1trans-11, ácido vaccénico; C18:2, ácido linoleico; C18:2cis-9,trans-11, ácido ruménico; C18:3, ácido linolénico.

En el rumen, el metabolismo de lípidos contenidos en la dieta comienza con la lipólisis por enzimas microbianas (lipasas, galactosidasas y fosfolipasas) producidas por bacterias como *Ruminococcus albus*, *Ruminococcus flavefaciens*, *Fibrobacter succinogenes* y algunas cepas de *Butirivibrio fibrisolvens*, entre otras, y diferentes especies de protozoos y hongos (Or-Rashid *et al.*, 2007). Las transformaciones ejercidas sobre triglicéridos, diglicéridos y galactolípidos conducen a la formación de dos o tres ácidos grasos y glicerol. La lipólisis suele ocurrir de forma rápida y casi total (90% en menos de una hora; Immig *et al.*, 1993). Sin embargo, un incremento de la concentración de almidón en la dieta reduce de forma muy significativa la tasa de lipólisis en el rumen. Otro factor que influye en la velocidad y el grado de lipólisis es la fuente de grasa, alcanzando un valor más alto en las grasas puras que en las protegidas que forman jabones cálcicos, o en las grasas que se integran en la estructura celular como las semillas oleaginosas enteras (Doreau y Ferlay, 1994). La cobertura (testa) de la semilla tiende a proteger los

lípidos dentro las semillas y hacerles menos accesibles a la hidrólisis ruminal comparado con la grasa de origen animal. Reddy *et al.* (1994) indicaron que el grado de hidrólisis del aceite de soja era mayor cuando se suministraba puro, que si se era añadido a la dieta en forma de haba de soja integral. También observaron que el procesado por extrusión del haba de soja aceleraba la liberación de ácidos grasos, como consecuencia de la ruptura de las membranas celulares y de la mayor disponibilidad del aceite (que está localizado intracelularmente) para los microorganismos.

La biohidrogenación de los ácidos grasos insaturados liberados en la lipólisis se lleva a cabo por los microorganismos ruminales y da lugar a isómeros trans y ácidos grasos saturados (AGS) (Carriquiry *et al.*, 2008). Este proceso es el resultado de la adición de hidrógeno (H) a los ácidos grasos con dobles enlaces. Aunque la mayoría de los ácidos grasos insaturados son modificados mediante el metabolismo ruminal, la saturación no suele ser completa normalmente, afectando entre el 70 y 90 % de los AG, y pueden aparecer diversos ácidos grasos como resultado de esta hidrogenación incompleta (Carro *et al.*, 1997).

Respecto a los ácidos grasos poliinsaturados, sólo el 10-15% de los consumidos en la dieta logran no ser biohidrogenados en el rumen (Givens *et al.*, 2006). El porcentaje de hidrogenación está en relación con la cantidad de ácidos grasos poliinsaturados que lleguen al rumen y del pH ruminal. A mayor cantidad de ácidos grasos insaturados, menor va a ser la proporción de biohidrogenación. Cuando más bajo es el pH ruminal, mayor es la inhibición del crecimiento de las bacterias encargadas de la biohidrogenación, sobre todo del grupo que realiza el último paso (de 18:1 a 18:0), quedando de esa forma mayor cantidad de metabolitos intermedios (Relling y Mattioli, 2003).

Los microorganismos ruminales no almacenan lípidos como triglicéridos, pero deben sintetizar sus membranas plasmáticas para lo cual emplean ácidos grasos que toman del rumen o bien que sintetizan en su citoplasma, creando así una variedad de ácidos grasos, algunos de ellos de cadenas impares y ramificadas (Zapata *et al.*, 2011). También modifican la longitud de la cadena de los ácidos grasos, tanto mediante alfa oxidación como beta oxidación. La síntesis de ácidos grasos por parte

de los microorganismos es generalmente moderada, aunque es mayor cuando la dieta contiene pocos lípidos, y puede aumentar con el consumo de concentrados. Las bacterias y los protozoos del rumen incorporan fácilmente ácidos grasos de la dieta a los lípidos celulares (Wu *et al.* 1991 a). Los ácidos grasos de los microorganismos son reciclados en el rumen tras la muerte bacteriana, representando un factor de crecimiento importante para otros microorganismos y siguiendo alguna de las vías de los demás ácidos grasos.

Los ácidos grasos que tienen menos de 12 carbonos son vertidos directamente a la vena porta y transportados al hígado unidos a la albúmina sérica. El resto de los lípidos que abandonan el rumen son predominantemente ácidos grasos saturados no esterificados de origen alimentario y microbiano (70%), y cantidades variables de fosfolípidos microbianos (10-20%) (Bauchart, 1993). El C18:1 total alcanza un valor medio de 6,2% en el que los isómeros *trans* representan algo menos de la mitad (Sauvant y Bas, 2001). En el intestino, estos ácidos grasos son esterificados e incorporados a lipoproteínas de muy baja densidad y quilomicrones para ser transportados por vía linfática hasta el torrente sanguíneo y distribuirlos a los tejidos.

Los ácidos grasos pueden tener diferentes destinos o usos metabólicos. Principalmente los ácidos grasos son asociados al metabolismo energético en el animal, por lo que los lípidos son sintetizados y depositados (lipogénesis) o degradados (lipólisis) en respuesta al balance energético del animal. La lipogénesis ocurre ante un balance energético positivo y comprende la síntesis de tres ácidos grasos y su esterificación con α-glicerofosfato para formar triacilglicéridos (TAG). La lipólisis predomina en el organismo al establecerse un balance energético negativo o bien en situaciones de estrés. Comprende la hidrólisis de los TAG y la liberación de los ácidos grasos y el glicerol desde los sitios de depósito. Además, los ácidos grasos también pueden cumplir una función estructural como fosfolípidos de membrana, pueden formar parte de un sistema de segundo mensajero como en el caso del fosfatidilinositol, etc.

El desarrollo de los depósitos adiposos en vacuno ocurre en el orden abdominal, intermuscular, subcutáneo y, finalmente, intramuscular (Pethick y Dunshea, 1996). La mayor actividad sintética ocurre en los depósitos abdominales de

los rumiantes jóvenes en crecimiento, mientras que la capacidad lipogénica subcutánea es mayor en los animales en cebo y los adultos (Bell, 1982). La adición de lípidos a las dietas de los rumiantes en cebo incrementa la proporción de grasa en la canal, independientemente de la fuente de grasa utilizada (Clinquart *et al.*, 1995).

La cantidad de triglicéridos almacenados en los depósitos grasos resulta del equilibrio entre la síntesis de novo y la captación de ácidos grasos preformados de la sangre para formar triglicéridos, y la lipólisis para la movilización de AGNE hacia la sangre o la reesterificación en el propio tejido (Chilliard, 1993; Figura 3). La esterificación de los ácidos grasos con el glicerol no ocurre en orden aleatorio: los AGS se sitúan en la posición sn-1, los ácidos grasos más cortos, los AGI, los ácidos grasos de cadena ramificada y los isómeros C18:1cis se encuentran mayoritariamente en la posición sn-2, y los restantes ácidos grasos de cadena larga y los isómeros C18:1trans ocupan la posición s*n-3* (Demeyer y Doreau, 1999).

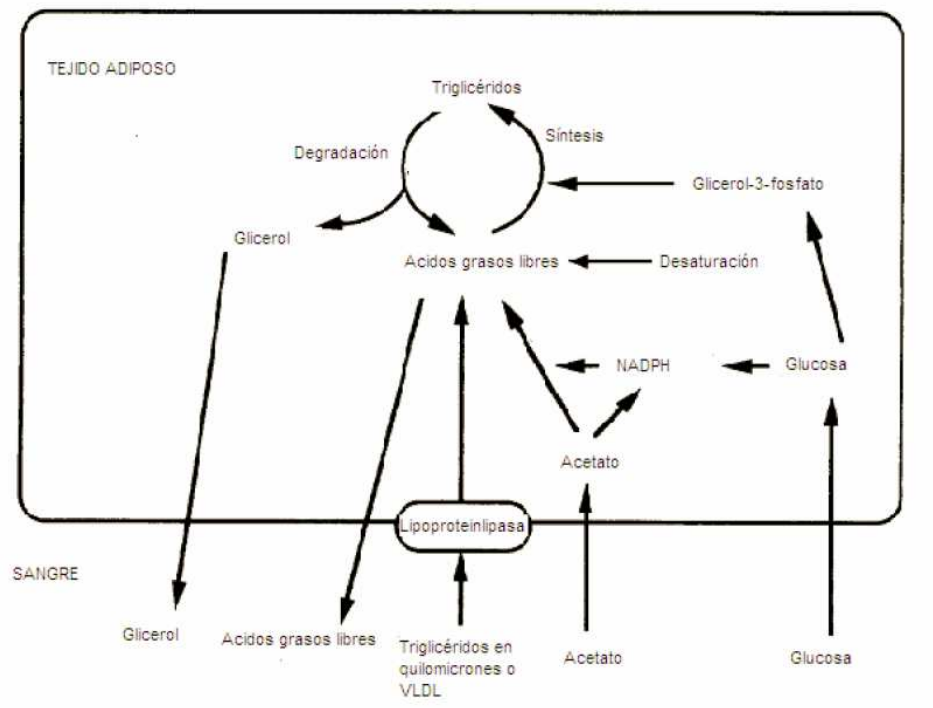


Figura 3. Metabolismo de los lípidos en el tejido adiposo de los rumiantes. Adaptado de Chilliard (1993).

2.2. Efecto de la utilización de ingredientes ricos en ácidos grasos poliinsaturados *n-3* y CLA en las dietas de cebo de terneros

2.2.1. Efecto de la utilización de ingredientes ricos en ácidos grasos n-3

La manipulación de la composición de la grasa en rumiantes para mejorar su perfil nutricional es uno de los aspectos claves en la investigación de la carne. Se quiere incrementar el contenido de los ácidos grasos esenciales *n-3* (α-linolénico y sus derivados EPA, DHA) y CLA, por los beneficios que presenta (Tabla X). Mientras que las recomendaciones nutricionales de la dieta global dicen que el ratio *n-6*/*n-3* debería estar por debajo de 4.0 (Departament of Health, 1994), la carne producida en España presenta una relación *n-6*/*n-3* alta, en algunos casos superior a 16 (Insausti *et al.*, 2004). El ratio *n-6*/*n-3* depende del contenido de las fracciones de fosfolípidos y triglicéridos, y se ve más afectado por la dieta que la genética animal (Mir *et al.*, 2003).

Tabla X. Beneficios para la salud de los ácidos grasos poliinsaturados *n-3*. Adaptado de Ganesan *et al.* (2014).

Beneficio	Ácido graso estudiado	Sujeto u organismo modelo	Referencia
Reduce la resistencia a la insulina	ALA	Humano	(Vuksan *et al.*, 2007)
Reduce la aterosclerosis	DHA, EPA	Humano	(Dyerberg *et al.*, 2004)
Ayuda al desarrollo neuronal y del cerebro	ALA, DHA	Humanos, roedores, otros primates	(Lauritzen *et al.*, 2000; McCann and Ames, 2005)
Antitumoral	DHA	Humano, rata	(Conklin, 2002; Holian and Nelson, 1992)
Previene la apoptosis	DHA, EPA	Rata	(Calviello *et al.*, 1999; German *et al.*, 2006)
Previene la inflamación	ALA	Ratón, rata	(Ren *et al.*, 2007)
Mejora la densidad ósea	DHA	Humano	(Hogstrom *et al.*, 2007)
Alivia la inflamación en la fibrosis quística	DHA, EPA	Humano	(De Vizia *et al.*, 2003)
Combate el estrés oxidativo	DHA	Gato, perro, humano	(Brown, 2008; Yavin *et al.*, 2002)
Contra la trombosis	EPA	Humano	(Tamura *et al.*, 1992)
Contra la arritmia	DHA, EPA	Humano	(Lombardi and Terranova, 2007; Nodari *et al.*, 2009)
Inmunomodulación	DHA, EPA	Humano	(Yaqoob and Calder, 2007)
Aumenta las funciones neuronales, cerebrales	DHA, EPA	Humano	(Chen *et al.*, 2008; German *et al.*, 2006; Lauritzen *et al.*, 2000; Valentine

Beneficio	Ácido graso estudiado	Sujeto u organismo modelo	Referencia
y de visión			and Valentine, 2004)
Mitiga mortalidad por enfermedades cardiovasculares	DHA, EPA	Humano	(GISSI, 1999)

Nota: ALA, ácido α-linolénico; EPA, ácido eicosapentanoico; DHA, ácido docosahexanoico

Una forma de aumentar la concentración de poliinsaturados en el tejido graso de la carne de vacuno es a través de productos protegidos contra la acción de las bacterias del rumen. Una gran variedad de procesos han sido estudiados, como el uso de aceites transformados por calor o procesos químicos, tratamientos químicos para formar jabones de calcio o amidas, emulsificación o encapsulación de aceites con proteínas y posterior protección química (Ashes *et al.*, 2000; Gulati *et al.*, 2005), además del empleo de plantas y semillas oleaginosas, aceites de pescado, algas marinas o suplementos de grasas (Givens *et al.*, 2000). Además de estudiar el enriquecimiento en ácidos grasos de la carne con estas técnicas, hay que tener en cuenta el posible efecto que puedan tener sobre la calidad del producto. Por ejemplo, dietas ricas en aceite de pescado mejoraron el perfil lipídico de la carne, pero también provocaron cambios indeseables en el sabor y la estabilidad de la carne de vacuno (Wood *et al.*, 2004).

Los aceites o semillas de plantas oleaginosas son importantes fuentes de *n-3*. En el caso de los rumiantes es más común el uso de semillas enteras, puesto que la cutícula del grano, actúa como protector frente a las bacterias del rumen, lo que hace que pueda llegar una mayor cantidad a los tejidos.

La semilla de lino (Figura 4) tiene alrededor de 40% de lípidos, 30% de fibra dietética y 20% de proteína. El aceite constituye el componente principal de la semilla de lino (35 a 43 g/100g materia seca).

Figura 4. Semillas de lino.

Esta composición puede variar entre las diferentes variedades de lino y en función de las condiciones ambientales en las que haya crecido la planta. En los cotiledones se encuentra el 87% de los lípidos y el 76% de la proteína de la semilla, en tanto que en el endosperma está sólo el 17% de los lípidos y el 16% de la proteína (Babu y Wiesenfeld, 2003; Daun *et al.*, 2003; Oohma, 2003). Las semillas de lino se caracterizan por un elevado contenido del ácido linolénico (Tabla X). Debido al reducido tamaño de las semillas de lino (2.5 – 6 mm), éstas resisten bien el proceso de rumia sin ser trituradas y su envuelta mucilaginosa reduce en gran medida la degradación del rumen. Las semillas son digeridas en el abomaso de forma que el ácido linolénico es absorbido, sin sufrir degradación microbiana, directamente en el intestino delgado e incorporado al flujo sanguíneo, lo que permite un máximo aprovechamiento del contenido de sus ácidos grasos y del linolénico en particular.

Tabla 2. Composición en ácidos grasos de las semillas de lino (Belitz *et al.*, 1997).

	Composición %
Ácido palmítico (C16:0)	6.5
Ácido esteárico (C18:0)	3.5
Ácido oleico (C18:1)	18
Ácido linoleico (C18:2)	14
Ácido linolénico (C18:3)	58

La adición de semilla de lino en dietas de terneros ha sido estudiada previamente por diferentes autores (Scollan *et al.*, 2001; Scollan *et al.*, 2003; Maddock *et al.*, 2006; Razminowicz *et al.*, 2008; Scholljegerdes and Kronberg, 2010; Kronberg *et al.*, 2011; Nassu *et al.*, 2011; He *et al.*, 2012; Kronberg *et al.*, 2012; Corazzin *et al.*, 2013; Mapiye, Aalhus, *et al.*, 2013; Mapiye, Turner, *et al.*, 2013; Albertí *et al.*, 2014), aumentando el total de *n-3* y la proporción y contenido de C18:*n-3* de la grasa intramuscular, así como el contenido en C20:5*n-3* y C22:5*n-3*. Asimismo, las relaciones *n-6*/*n-3* disminuyeron en las carnes procedentes de animales alimentados con las dietas enriquecidas con lino.

Aunque muchos trabajos han estudiado el efecto de la adición de lino en la dieta sobre la variación de la deposición de los ácidos grasos de la grasa intramuscular y subcutánea, pocos han sido los trabajos que han estudiado cómo afecta esta variación a la calidad de la carne y a la alteración de su vida útil. Los AG están involucrados en varios aspectos tecnológicos de la calidad de la carne, por lo que la modificación de la composición de la grasa, aumentando la proporción de los ácidos grasos poliinsaturados, puede comportar alteraciones en la calidad del producto. Estas alteraciones comprenden desde la aceleración de la oxidación lipídica y aparición de olores y sabores a rancio, hasta la alteración del color de la carne debido a la oxidación de los pigmentos musculares, procesos que acortarían la vida útil de la carne (Wood *et al.*, 2003).

Mach *et al.* (2006) informaron que el tipo de grasa de la dieta (colza o lino) o su nivel de inclusión (5% a 11%) no afectaron las características de la canal, ni el color de la carne. Asimismo, Albertí *et al.* (2014), que adicionaron 5 % semilla de lino en dietas de terneros de raza Pirenaica, no encontraron efectos negativos sobre la estabilidad del color de la carne durante su almacenamiento. Estos resultados implicarían que la calidad de la carne no sufre siempre modificaciones negativas al incluir una fuente de ácidos grasos poliinsaturados. Por tanto, la semilla de lino ofrecería la oportunidad del enriquecimiento de la carne en ácidos grasos *n-3*, aunque habría que profundizar en los estudios sobre la alteración de la vida útil y la aptitud tecnológica de las carnes enriquecidas.

1.2.2. Efecto de la utilización de ingredientes ricos en ácidos grasos CLA

El ácido linoleico conjugado (CLA) es un ácido graso que, en las últimas dos décadas, ha llamado la atención significativamente por sus beneficios potenciales para la salud humana (Decker y Park, 2010).

El CLA (cis-9, cis-12), un ácido graso omega-6, es muy abundante en el reino vegetal y en la grasa animal y representa una mezcla de isómeros geométricos y de posición del ácido linoleico (cis-9, cis-12, ácido octadecadienoico) con dobles enlaces conjugados. Los pares de enlaces dobles del ácido graso poseen, generalmente, un grupo metileno entre ellos, pero el CLA presenta un enlace conjugado.

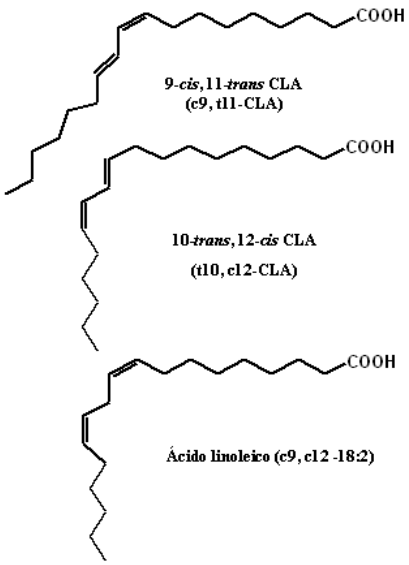


Figura 5. Estructura de los isómeros de CLA cis-9, trans-11; trans-10, cis-12; y ácido linoleico cis-9, cis12. (García–Solís *et al.*, 2005).

Teóricamente, muchos isómeros de CLA pueden diferir en las posiciones del enlace doble (por ejemplo, 7-9, 8-10, 9-11, 10-12, y así sucesivamente). Los dos isómeros de CLA, más estudiados son cis-9, trans-11 y trans-10, cis-12. En la figura 5 se puede observar cómo sus estructuras se diferencian del ácido linoleico, un ácido graso dietético esencial, que también contiene 18 carbonos y 2 enlaces dobles. Entre los isómeros de CLA pueden existir diferencias adicionales en la configuración del doble enlace, como los cis-trans, trans-cis, cis-cis o trans-trans, siendo todos posibles.

El CLA presenta diferentes isómeros; cis-7, trans-9; cis-9, trans-11; cis-11, trans-13 principalmente, (Fritsche y Steinhart, 1998), pero la forma biológicamente activa y más importante de los CLA está representada por el isómero *cis-9, trans-11* CLA (Chin *et al.*, 1992; Kramer *et al.*, 1998), constituyendo un ácido graso capaz de inhibir la carcinogénesis en animales experimentales (Parodi, 1999). No obstante, cis-11, cis-9 ha sido indicado como un isómero más efectivo contra células cancerígenas mamarias (Tanmahasamut *et al.*, 2004), pero su presencia en la leche y carne de rumiantes se ha informado raramente (Khanal y Olson, 2004). Otro isómero de importancia cuantitativa, para el cual su papel biológico es desconocido, es cis-9,

trans-7 que supone entre el 3 y el 16% de CLA total en productos rumiantes (Yurawecz *et al.*, 1998).

Los compuestos CLA, en animales rumiantes, representan productos que se forman por dos vías (Figura 6) (Corl *et al.*, 2003; Kay *et al.*, 2004): la incompleta biohidrogenación ruminal del ácido linoleico (C18:2n6,cis9,cis12) a ácido estéarico (C18:0) (Bauman *et al.*, 2001), por la bacteria *Butyrivibrio fibrisolvens* (Kepler *et al.*, 1966) y otras bacterias del rumen (Kritchevsky, 2000); y la conversión endógena del ácido transvacénico (C18:1,t11, VA) por la enzima delta-9 desaturasa en los tejidos (Corl *et al.*, 2001).

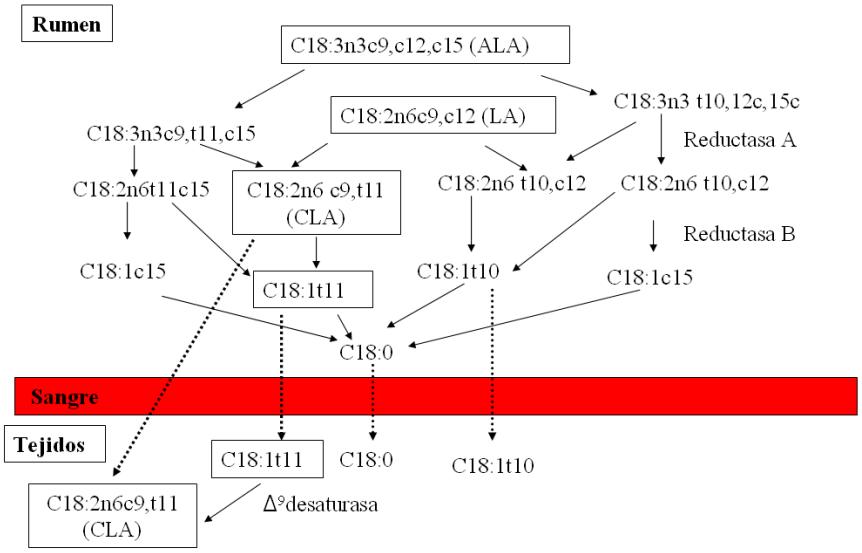


Figura 6. Biohidrogenación del ácido linoleico y linolénico por enzimas bacterianas del rumen y síntesis endógena del ácido linoleico conjugado (modificado de Kramer *et al.*, 2004).

El descubrimiento de CLA como un "alimento funcional" ocurrió hace años cuando Pariza *et al.*, (2001) encontraron en la carne un factor anti-mutagénico que consistía en una serie de isómeros conjugados del ácido linoleico. Luego, varios estudios demostraron que el CLA sintetizado químicamente podía reducir la incidencia de varios tipos de tumores en modelos animales. Pero la mayoría de los agentes naturalmente anti-cancerígenos se encontraban presentes sólo en niveles traza y eran de origen vegetal. Sin embargo, el CLA era el único entre los agentes anti-cancerígenos naturales que presentaba potentes efectos en niveles

extremadamente bajos y que se encontraba presente en los productos lácteos y la carne de animales rumiantes.

La bioactividad más significativa del isómero *cis-9, trans-11* CLA es su propiedad contra el cáncer, como se muestra en una serie de modelos animales de cáncer como el de mama, epidermis, próstata, colon, hígado, riñón y pulmón (Ip *et al.*, 1991; Lee *et al.*, 2005; Bhattacharya *et al.*, 2006; Kelley *et al.*, 2007; Park, 2009). Además de las propiedades anticancerígenas presenta otras propiedades positivas para la salud, como la reducción en el aumento de las grasas corporales, retraso en el inicio de la diabetes tipo II, retardo en el desarrollo de la arteriosclerosis, mejoramiento de la mineralización de los huesos y modulación del sistema inmune (Pariza *et al.*, 2001; Belury, 2002; Whigham *et al.*, 2000; Park y Pariza, 2007). Todo ello ha dado lugar a un aumento exponencial en la investigación acerca de los efectos del CLA en los últimos años, realizándose estudios para conocer su efecto sobre el síndrome metabólico (Nagao y Yanagita, 2008; Yanagita y Nagao, 2008), el asma (Jaudszus y Jahreis, 2007), la calidad de los huesos (Shen y Yeh, 2007), y la enfermedad de Crohn (Gilman y Cashman, 2007). Se ha sugerido que el isómero trans-10, cis-12 causa una reducción en la deposición de lípidos en animales en crecimiento (de Deckere *et al.*, 1999).

Aunque lo fisiológicamente importante es el balance dietético de los AG en la dieta total, se ha intentado cambiar la composición de los alimentos de forma individual de acuerdo a las nuevas recomendaciones nutricionales (Wood y Enser, 1997).

Los productos de la leche y de la carne de rumiantes representan la fuente natural más importante del isómero *cis-9, trans-11* CLA, representando alrededor de un 90% en la leche y un 75% en la carne de vacuno (Chin *et al.*, 1992), siendo además su concentración en los tejidos adiposos de vacuno de especial interés para la salud humana (Varhegyi y Varhegyi, 2007). Por otra parte, existe potencial para enriquecer el contenido de la carne con CLA y AV a través del aumento de la disponibilidad del sustrato en la dieta animal, el ácido linoleico. El AV proveniente de la carne es una fuente importante de CLA debido a que los humanos y otras

especies animales presentan la capacidad de producir CLA a partir del AV (Dunshea *et al.*, 2005).

Existen diversos estudios sobre los efectos de la adición de CLA en la dieta para aumentar el rendimiento de los animales, mejorar la calidad de la carne y ofrecer productos cárnicos con alto contenido de CLA. Los resultados obtenidos en estos estudios son a veces contradictorios, lo cual podría explicarse por los distintos factores que se incluyen en cada estudio, como la especie animal, raza, edad, duración de la administración y niveles de CLA, condiciones de cría y composición de los piensos (Zhang *et al.*, 2010).

La suplementación animal con aceites de pescado aumenta los niveles de AG *n-3* en carne (Wood *et al.*, 2004) y puede aumentar la producción de CLA y AV (AbuGhazaleh *et al.*, 2003; Shingfield *et al.*, 2003) reduciendo la conversión ruminal del AV a ácido esteárico (Griinari y Bauman, 1999; Lourenco *et al.*, 2008). Sin embargo, dietas ricas en aceite de pescado provocan cambios indeseables en el sabor y la estabilidad de la carne (Wood *et al.*, 2004; Wistuba *et al.*, 2006).

Por otra parte, la alimentación animal con CLA protegido también ofrece la posibilidad de aumentar el contenido de CLA en la carne de rumiantes (Gillis *et al.*, 2004; Richardson *et al.*, 2006). La investigación en ganado vacuno de leche ha mostrado que la suplementación con sales de CLA o aceites vegetales aumentan el isómero de CLA cis-9, trans-11 en la grasa de la leche (Kelly *et al.*, 1998; Corl *et al.*, 2001).

También existen estudios que han evaluado el efecto de la inclusión de CLA protegido sobre el perfil de AG en la carne (Gillis *et al.*, 2004; Poulson *et al.*, 2004; Gillis *et al.*, 2007; Schiavon *et al.*, 2011; Schlegel *et al.*, 2012), pero pocos analizan el efecto sobre las alteraciones en la vida útil y aptitud tecnológica de las carnes.

Richardson *et al.* (2006) y Fagali y Catala, (2008) propusieron que el CLA podría mejorar la estabilidad de los lípidos indicando valores inferiores de TBARS en la carne proveniente de animales alimentados con concentrado y CLA protegido, sugiriendo que el CLA se oxidaba preferentemente previniendo así la oxidación de otros AG.

3. Aspectos relacionados con la carne

La denominación genérica de carne, según el Código Alimentario Español, incluye la parte comestible de los músculos de los bóvidos, óvidos, cápridos, équidos y camélidos sanos, sacrificados en condiciones higiénicas. Por extensión, se aplica también a la de los animales de corral, caza de pelo y pluma, y mamíferos marinos. Presentará un olor característico, y su color debe oscilar del blanco rosáceo al rojo oscuro, dependiendo de la especie animal, raza, edad, alimentación, forma de sacrificio y periodo de tiempo transcurrido desde que aquél fue realizado.

Los valores medios aproximados para la composición bruta y el contenido energético de la fracción comestible de la carne fresca son: 17% de proteína, 20% de grasa, 62% de humedad, 1% de cenizas y 250 kcal/100 g (valores adecuados para carnes con un recubrimiento graso de aproximadamente 1 cm de espesor). Los trozos de músculo magro son más uniformes en composición: 20% de proteína, 9% de grasa, 70% de humedad, 1% de cenizas y 160 kcal/100 g. La carne comercial no posee prácticamente carbohidratos (menos del 1%), ni tampoco contiene fibra (Price y Schweigert, 1994).

La calidad de la carne puede verse afectada por numerosos factores que pueden dar lugar a oxidaciones lipídicas y proteicas, por lo que conseguir un mayor grado de homogeneidad en los productos, es una de las mayores preocupaciones de la industria cárnica, lo que requiere un estudio de los factores que afectan y definen a la calidad de la carne.

3.1. Calidad de la carne

En términos generales, la composición de la carne se establece completamente durante la vida del animal, mientras que su calidad se ve fuertemente afectada por factores tanto *ante mortem* como *post mortem*. Todos los procesos que se producen tras el sacrificio son de gran importancia, porque la canal es mucho más susceptible que el animal vivo a tratamientos que puedan fomentar sus atributos de palatabilidad.

La calidad es un término muy complejo que tiene diversas acepciones dependiendo de cuál sea la etapa del proceso (producción, comercialización, etc.) en que nos encontremos. La calidad higiénica es lo primero que debe tener la carne, libre de agentes bacterianos y de residuos que constituyan un riesgo para el consumo de esa carne (Gracey, 1989). Existe una legislación al respecto con unos parámetros mínimos de calidad. La calidad bromatológica hace referencia al valor nutritivo de la carne. La calidad tecnológica se relaciona con las propiedades de la carne que determinan su aptitud para la transformación y conservación (Dikeman, 1991). También existen otras acepciones como la calidad simbólica, relacionada con prohibiciones religiosas, imágenes ligadas a campañas publicitarias, etc., o la calidad de presentación, que hace referencia a las modificaciones de los cortes tradicionales, a nuevos productos con nuevas presentaciones, etc., que pueden variar la intención de compra (Sañudo, 1992). La calidad organoléptica o sensorial (Romans y Norton, 1989; Ingr, 1990; Wal, 1991; Boccard, 1992) puede definirse como el conjunto de las características percibidas por los sentidos en el momento de la compra o del consumo, que influyen en la satisfacción sensorial (Sañudo, 1992).

La calidad organoléptica viene dada por unos parámetros enormemente variables, fácilmente modificables, objetivos y mensurables, intrínsecos a la propia naturaleza de la carne, y determinantes en el momento clave de todo proceso productivo – tecnológico, es decir, en el momento de la compra – consumo. Las características organolépticas que van a influir en la palatabilidad de la carne son, fundamentalmente, la textura, la jugosidad, el flavor, y el color. Por su parte, estos atributos se hallan influidos, como ya se ha mencionado, por los factores productivos y tecnológicos ya explicados anteriormente.

Atendiendo a las distintas definiciones de calidad, existen diversos parámetros y atributos indicativos de la calidad de la carne, como son, el pH, el color, el contenido en pigmentos, la flora bacteriana, la capacidad de retención de agua, la composición química y energética, los niveles de oxidación lipídica, propiedades de textura, atributos sensoriales como olor, gusto, aromas percibidos durante la masticación, etc. Dichos atributos de calidad no pueden considerarse independientes, ya que están muy relacionados entre sí y su interacción proporciona las características globales de calidad de carne.

3.1.1. Factores que afectan a la calidad de la carne

La obtención de los parámetros de calidad está determinada por todos y cada uno de los eslabones que intervienen en la producción de la carne, como son el ganadero, el matadero, la comercialización y el consumidor. La producción y obtención de la carne es un sistema complejo en el que intervienen aspectos tanto productivos como tecnológicos (Figura 7).

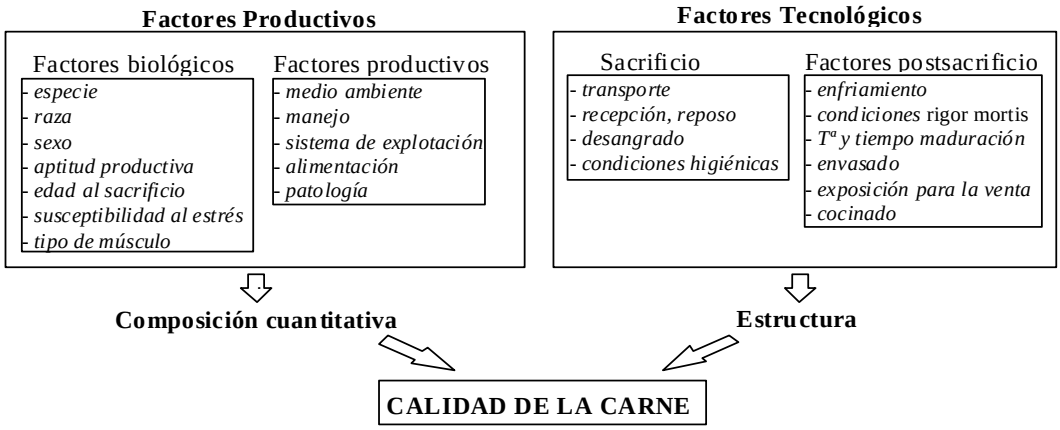


Figura 7. Factores que influyen en la calidad de la carne (Beriain y Lizaso, 1997).

La calidad de la carne depende de diversos factores intrínsecos propios del animal (raza, genotipo, sexo y edad) y extrínsecos o ligados al proceso productivo (alimentación, castración), además de otros relacionados con el manejo del animal y la canal en los momentos previos y posteriores al sacrificio (transporte, tiempo de espera, ayuno, estrés, método de aturdimiento, sangrado, enfriamiento de la canal, tiempo de maduración, envasado, etc.). Dichos factores pueden dividirse en función del espacio temporal en el que actúan en factores:

- *Ante mortem*: raza, hipertrofia muscular, sexo, edad y alimentación.
- *Peri mortem*: transporte, manejo y estrés al sacrificio.
- *Post mortem*: enfriamiento, envasado, maduración, estimulación eléctrica, congelación, conservación y métodos de cocinado.

La raza es un factor muy importante que afecta a muchas características productivas de los animales y también a la calidad final de la carne. Se han realizado

diversas clasificaciones de distintas razas de ganado vacuno atendiendo a criterios productivos (razas cárnicas *vs* razas lecheras), a la velocidad con la que alcanzan la madurez, lo que determinará su edad óptima de sacrificio (maduración rápida o lenta), al tamaño corporal, etc.

Las diferencias entre sexos están bien definidas: a la misma edad, las hembras tienen la carne más tierna que los machos, y los machos castrados son más tiernos que los enteros (Field, 1971; Misock *et al.*, 1976; Touraille y Girard, 1985). No obstante, algunos autores contradicen estos resultados, puesto que no encontraron diferencias significativas entre sexos (Kemp *et al.*, 1981; Sañudo *et al.*, 1986; López, 1987). Generalmente la carne de las hembras es de mejor calidad sensorial que la de los machos (Riggs *et al.*, 1967; Purchas *et al.*, 2002).

El efecto de la edad del animal sobre la calidad de la carne aún no está claro debido a que, en muchas ocasiones, el estudio del efecto edad interacciona con otros factores como la velocidad de crecimiento del animal y el nivel de alimentación (Purchas *et al.*, 2002), por tanto, es preciso diferenciar entre la edad cronológica (días desde el nacimiento) y la edad fisiológica (porcentaje de peso vivo adulto alcanzado) que determina el estado de desarrollo del individuo, ya que esta última influye en la diferencia entre razas, determinando su precocidad y su peso al sacrificio (Santolaria, 1993).

La alimentación es uno de los factores que más influyen en la calidad final de la carne, sobre todo debido a que la nutrición puede tener un efecto regulador sobre los procesos biológicos que tienen lugar en el músculo y que finalmente determinarán la calidad del producto (Andersen *et al.*, 2005; Descalzo *et al.*, 2007). Se ha demostrado que la dieta afecta a la composición química de la carne de vacuno (O'Sullivan *et al.*, 2003), la composición de la grasa (Descalzo *et al.*, 2005; Descalzo *et al.*, 2007), contenido en colágeno (French *et al.*, 2001; Archile *et al.*, 2010), color de la carne y de los tejidos adiposos (Priolo *et al.*, 2001; Dunne *et al.*, 2006) y la calidad sensorial (Warren *et al.*, 2008).

La carne lista para el consumo se obtiene después de un cierto tiempo de almacenamiento en refrigeración (0-5°C) conocido como periodo de maduración, tras el cual la carne resulta más tierna y jugosa (Carballo y López de Torre, 1991). La

maduración habitual de la carne se realiza por almacenamiento en frío de los medios o cuartos de canal durante 10 ó 14 días. Sin embargo, su vida útil está limitada principalmente por dos factores: el efecto del oxígeno atmosférico y el crecimiento de microorganismos aerobios productores de alteraciones. Estos factores, de forma individual o asociados con otros, producen cambios de olor, sabor, color y textura, que van produciendo un deterioro general de la calidad. El almacenamiento refrigerado retrasa estos cambios indeseables, pero no incrementa la vida útil lo suficiente para las exigencias actuales de la distribución al por menor. Existen diversos tipos de envasado en atmósferas protectoras para incrementar la vida útil de los alimentos. El método de referencia en la mayoría de las investigaciones es el envasado al vacío, que permite estudiar períodos largos de maduración *post mortem* y se emplea ampliamente para productos como primeros cortes de carnes rojas frescas (López Vázquez y Vanaclocha, 2004). En estas condiciones se inhibe la proliferación de patógenos y alterantes aerobios y la oxidación lipídica. Además, este sistema facilita la manipulación y transporte de la carne.

El tiempo de maduración de la carne es fundamental para la adquisición de un grado de terneza adecuado debido al ablandamiento de la carne, que se atribuye a una degradación progresiva y selectiva de la estructura de las miofibrillas a causa de la acción de enzimas proteolíticos endógenos. Además, a lo largo de la maduración ocurren fenómenos oxidativos que afectan a lípidos y proteínas y provocan cambios en el color de la carne y contribuyen de forma positiva en el desarrollo adecuado de su flavor característico. Marino *et al.* (2006) comprobaron que una extensión del tiempo de maduración de 15 a 21 días *post mortem* incrementaba el flavor de la carne, coincidiendo con resultados de Campo *et al.* (1999) y Napolitano *et al.* (2001) que encontraron que la intensidad del flavor se incrementaba con el tiempo de maduración, probablemente debido a fenómenos de proteolisis y lipolisis que dan lugar a la formación de precursores del sabor.

La vida útil es el máximo tiempo de almacenamiento antes de que la carne pierde su calidad nutricional, sensorial y de seguridad alimenticia que haga que dicha carne sea rechazada por los consumidores (Masana *et al.*, 2006). En este sentido, las características microbiológicas y organolépticas del producto son determinantes. Los factores de conservación principales que afectan a la calidad de la carne y productos

cárnicos son la temperatura, el tiempo de mantenimiento, la iluminación, la exposición al oxígeno del aire, así como el tipo de envasado y el uso de atmósferas modificadas.

El cocinado de la carne es un factor de gran importancia pues influye en muchas características de su calidad. El calor altera el tejido conectivo y las proteínas miofibrilares, y de este modo puede influir significativamente en la dureza de la carne, en su jugosidad y en su sabor. Durante el cocinado se producen dos cambios fundamentales: las fibras musculares se hacen más duras por coagulación, y el tejido conectivo se hace más blando, por conversión del colágeno en gelatina (Lawrie, 1966; Davey y Gilbert 1974; Harris y Shorthose, 1988). El efecto endurecedor de las fibras y el ablandador del colágeno dependen del tiempo y de la temperatura (Dransfield, 1977). El color también se ve afectado por el cocinado, convirtiéndose en marrón según progresa el calentamiento.

3.2. Oxidación de la carne

La oxidación lipídica y la oxidación de la mioglobina en la carne dan lugar al desarrollo de flavores anómalos y a la decoloración, respectivamente. Estos procesos a menudo parecen estar vinculados y la oxidación de uno de éstos lleva a la formación de especies químicas que pueden potenciar la oxidación del otro (Faustman *et al.*, 2010).

3.2.1. Oxidación proteica

El término oxidación proteica hace referencia a la modificación de una proteína inducida de forma directa por especies reactivas derivadas del oxígeno.

Las principales consecuencias de la oxidación proteica sobre la calidad de la carne y productos cárnicos se aprecian sobre todo a nivel del color de la carne.

La química del color de la carne se debe fundamentalmente al estado de la proteína muscular mioglobina, por lo tanto aquellos músculos con un contenido elevado de este pigmento sufrirán las consecuencias de la oxidación proteica en mayor medida.

La mioglobina es la principal responsable del color rojo del músculo y tiene como función el almacenamiento del oxígeno necesario para el metabolismo aeróbico. Es una proteína conjugada constituida por una parte proteica (globina) y un grupo prostético de naturaleza no peptídica (grupo hemo). La molécula de mioglobina da a la carne fresca un color rojo púrpura. En contacto con el aire la molécula se oxigena dando lugar a la oximioglobina de color rojo brillante. Cuando la mioglobina se oxida se genera la forma férrica (Fe^{3+}), denominada metamioglobina, responsable de la coloración marrón de la carne. Por lo tanto, en presencia de oxígeno, la mioglobina se convierte en dos pigmentos diferentes, oximioglobina y metamioglobina, formas oxigenada (rojo brillante) y oxidada (marrón), respectivamente. Las proporciones relativas de estas dos formas en la carne dependen de la presión parcial de oxígeno, siendo favorecida la formación de metamioglobina por presiones de oxígeno bajas (Bodwell y McClain, 1971).

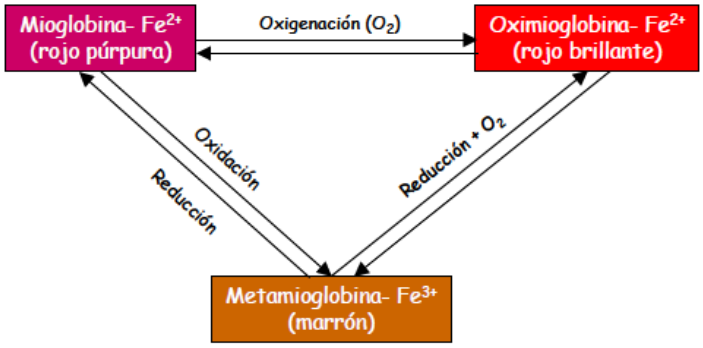


Figura 8. Pigmentos hemo del músculo.

3.2.2. Oxidación lipídica

La oxidación lipídica y los cambios asociados a ella constituyen la principal causa de deterioro de la calidad de la carne y de los productos cárnicos (Sevanian y Hochstein, 1985).

A través de la manipulación de la composición de los ácidos grasos suministrados en la dieta animal es posible modificar el perfil lipídico de los animales. Esto permite ajustar la cantidad y composición de estos lípidos a las necesidades humanas: reducir el nivel de saturación, aumentar el contenido en ácidos mono y poliinsaturados o disminuir la relación *n-6*/*n-3*. Pero uno de los principales

problemas que aparecen al aumentar el grado de instauración de la carne es el incremento de la susceptibilidad de ésta a la oxidación lipídica, especialmente durante los procesos de almacenaje y cocción (Jiménez-Colmenero, 2001c; Barroeta y Cortinas, 2002a).

La oxidación lipídica provoca la aparición de olores y flavores extraños, la alteración del color y, en general, una reducción de la calidad organoléptica de la carne. Por otro lado, provoca una disminución del valor nutritivo de la carne y la generación de compuestos potencialmente nocivos para la salud que se han relacionado con el riesgo de padecer diversas patologías (enfermedades cardiovasculares, cáncer, envejecimiento...). Todos estos factores contribuyen a disminuir la aceptación del producto por parte del consumidor (Barroeta y Cortinas, 2002b). Por lo tanto, si se desea aumentar el grado de insaturación de la carne es necesario encontrar un equilibrio que permita un aumento razonable en ácidos grasos poliinsaturados con el mínimo perjuicio organoléptico y oxidativo (Barroeta y Cortinas, 2002a).

a) Mecanismo de la oxidación lipídica

Los sustratos de las reacciones de oxidación lipídica son básicamente los ácidos grasos insaturados, formándose como productos peróxidos lipídicos (también llamados hidroperóxidos), polímeros, epóxidos, furanos, alcoholes, hidrocarburos y carbonilos (aldehídos y cetonas) volátiles.

Desde un punto de vista químico, la oxidación lipídica es una reacción en cadena de radicales libres que se da en tres etapas que, salvo en su comienzo, se desarrollan simultáneamente (Cheftel y Cheftel, 1980): iniciación, propagación y terminación (Figura 9). Mediante esta reacción en cadena, un radical puede inducir la oxidación de un número elevado de moléculas de sustrato (Simic, 1981; Halliwell y Gutteridge, 1989).

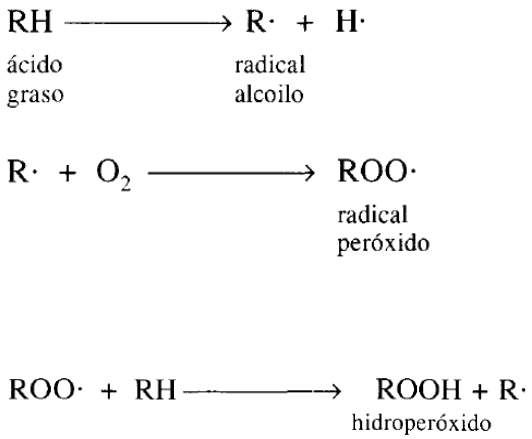


Figura 9. Reacciones químicas que se producen durante la oxidación.

Las reacciones de iniciación dan lugar a la formación de radicales libres a partir de ácidos grasos insaturados o de hidroperóxidos, que son sustancias muy inestables y reactivas. La oxidación comienza en el ácido graso insaturado con la pérdida de hidrógeno en el átomo de carbono α-metilénico dando lugar a un radical libre.

En las reacciones de propagación, los radicales libres se combinan con oxígeno para formar radicales peróxidos, que pueden oxidar otros ácidos grasos insaturados para producir hidroperóxidos insaturados y nuevos radicales libres, de forma que se propaga la reacción y se acumulan peróxidos lipídicos.

Por último, en las reacciones de terminación o paralización, los radicales libres, procedentes en gran parte de la descomposición de peróxidos lipídicos, se asocian para dar compuestos no radicales como aldehídos y cetonas, de bajo peso molecular, responsables del típico olor “a rancio”. Algunos de estos compuestos provienen directamente de la degradación de peróxidos.

Los hidroperóxidos formados en las reacciones de propagación son los productos primarios de la oxidación. Estos compuestos no tienen repercusión directa en el aroma de los alimentos, pues son inodoros e insípidos, pero sí intervienen en él a través de su descomposición en productos secundarios que son los principales responsables de los cambios organolépticos. Cada ácido graso insaturado da lugar a radicales libres específicos de los que resultan hidroperóxidos isómeros específicos que, al descomponerse producen también aldehídos específicos.

Los hidroperóxidos del ácido linolénico se descomponen más rápidamente que los de los ácidos oleico y linoleico debido a la presencia de grupos metileno activos; son los que se localizan entre un doble enlace simple y un grupo dieno conjugado y pueden perder el hidrógeno fácilmente para formar dihidroperóxidos, lo que amplía el abanico de productos de la oxidación (deMan, 1992).

Por lo general, los ácidos grasos insaturados se oxidan más rápidamente cuando están libres que cuando forman parte de moléculas de triglicéridos o fosfolípidos (Cheftel y Cheftel, 1980). Los AGP son mucho más susceptibles de oxidación que los monoinsaturados. Dicha susceptibilidad es mayor que la que cabría esperar en función del número de dobles enlaces de la cadena. Los AGP se oxidan incluso durante el almacenamiento de los alimentos en congelación. Por el contrario, los ácidos grasos saturados se oxidan sólo a temperaturas superiores a 60º C (Cheftel y Cheftel, 1980).

b) Oxidación lipídica en la carne y productos cárnicos

El proceso de oxidación de los lípidos se ha examinado de forma exhaustiva en la carne (Decker y Xu, 1998; Faustman *et al.*, 2010; Monahan, 2000). Una variedad de propiedades intrínsecas y de etapas de procesado pueden predisponer a la carne a la oxidación lipídica.

Aspectos relacionados con el animal como la especie, el sexo o la edad influyen en la susceptibilidad de la carne a la oxidación lipídica (Gray y Pearson, 1987; Kim *et al.*, 2002).

La composición de las fibras musculares, las áreas fibrilares y la densidad capilar del músculo son factores importantes que afectan a los procesos bioquímicos *ante y post mortem* y, por lo tanto, a la calidad de la carne (Klont *et al.*, 1998). Además, dentro de una misma especie animal, el contenido lipídico varía en función del músculo (Barbut, 2002a).

La composición de la grasa de la dieta y la tendencia de las especies a acumular ácidos grasos en los fosfolípidos de membrana, afectan a la composición lipídica de la membrana muscular y a su susceptibilidad a la oxidación (Kanner, 1994). Esta susceptibilidad dependerá del número de insaturaciones de los AG y

parece ser que aumenta de forma exponencial con la presencia de dobles enlaces en la molécula.

En principio, los productos cárnicos crudos son menos susceptibles a la oxidación que los tratados por el calor, ya que en estos últimos el calentamiento provoca una rotura de la estructura del tejido muscular causando la desnaturalización de proteínas y la consiguiente pérdida de actividad enzimática de algunas de ellas, además de liberar hierro que actúa como catalizador de la oxidación (Rhee *et al.*, 1987; Decker y Xu, 1998). El tratamiento térmico afecta a la actividad de enzimas antioxidantes (Mei *et al.*, 1994; Lee *et al.*, 1996b), se libera oxígeno de la oximioglobina que genera peróxido de hidrógeno (Harel y Kanner, 1985) y se produce la rotura de hidroperóxidos generando radicales libres que propagan la peroxidación (Kanner, 1994).

En la carne deshidratada los procesos oxidativos son mucho más lentos que en la carne fresca debido a su menor contenido de agua.

Los fenómenos de oxidación están favorecidos en los productos cárnicos por las distintas operaciones de procesado y condiciones de manipulación (picado, refrigeración y congelación repetidas, temperaturas elevadas y almacenamiento prolongado), así como por algunos de los ingredientes añadidos.

Muchos productos cárnicos, durante su proceso de fabricación, experimentan un cierto grado de modificación de la estructura muscular. En emulsiones, carne picada, troceada o productos cárnicos reestructurados, los diferentes grados de manipulación física producen roturas en la estructura y exponen a los lípidos del músculo a un ambiente prooxidante favoreciéndose el contacto entre los sustratos de oxidación y los catalizadores de ésta. Incluso en algunos casos puede detectarse un cierto olor a “sobrecalentamiento” en carnes crudas picadas, lo que apoya la teoría de que la rotura de la estructura muscular durante el procesamiento contribuye de forma importante a la oxidación lipídica (Decker y Xu, 1998; Monahan, 2002).

Otra variable relativa al procesado que influye en el grado de oxidación de la grasa de los productos cárnicos es el pH. A medida que su valor desciende por debajo de 7,0, la velocidad de oxidación es mayor (Tichivangana y Morrissey, 1985).

Por otro lado, ciertos ingredientes usados en la fabricación de los productos cárnicos afectan a su susceptibilidad a la oxidación (Monahan, 2002). El cloruro sódico puede promover la oxidación lipídica posiblemente por el desplazamiento de hierro unido a macromoléculas (Kanner *et al.*, 1991), a la vez que puede afectar a la actividad de los enzimas antioxidantes (Lee *et al.*, 1996b; Hernández *et al.*, 2002; Sárraga *et al.*, 2002). El nitrito, usado en la fabricación de carnes curadas, juega un papel importante en el desarrollo del color y en la preservación de la carne curada de la aparición de *Clostridium spp.* Su efecto antioxidante se ha atribuido a la generación de óxido nítrico que interacciona con el hierro evitando que este actúe como catalizador, a su capacidad para capturar radicales libres y a su capacidad para estabilizar ácidos grasos insaturados de las membranas celulares (Kanner, 1994; Monahan, 2002). Los fosfatos, ampliamente utilizados en los productos cárnicos para aumentar los enlaces con moléculas de agua, también funcionan como antioxidantes a través de la quelación de metales. Finalmente, los ascorbatos pueden tener efectos antioxidantes en productos cárnicos dependiendo de la cantidad en que sean añadidos (Monahan, 2002).

El conocimiento de las interacciones complementarias de la oxidación proporciona una base para explicar el deterioro de la calidad en la carne y también para desarrollar estrategias que mantengan la óptima calidad sensorial (Faustman *et al.*, 2010).

3.3. El consumidor

En general, los consumidores consideran la carne como un componente importante y saludable de la dieta (Verbeke *et al.*, 2010). Los cambios en la demanda de los consumidores de la carne y productos cárnicos, así como la creciente competencia global, están provocando un estímulo sin precedentes en la evolución del sistema de procesamiento y de los ingredientes dentro del sector de la carne. Los consumidores exigen productos más sanos que sean bajos en sal, grasa, colesterol, nitritos y calorías en general, y que contengan, además, componentes bioactivos promotores de la salud, como por ejemplo los carotenoides, ácidos grasos

insaturados, esteroles y fibras. Por otro lado, los consumidores esperan que estos nuevos productos cárnicos con formulaciones modificadas tengan gustos, aspectos y olores similares que los procesados y formulados de manera tradicional. Al mismo tiempo, la competencia está forzando a la industria de procesado de carne a utilizar materia prima cada vez de mayor valor comercial, obtener derivados cárnicos más eficientes y producir los productos a costos más bajos (Weiss *et al.*, 2010). Sin embargo, existe una resistencia de algunos consumidores a la incorporación de aditivos en los alimentos, especialmente cuando los aditivos son de origen sintético, incluso cuando tienen una ventaja nutricional o de salud. La modificación de la dieta de los animales proporciona un método único de manipular el contenido de algunos micronutrientes y otros compuestos bioactivos en la carne, con el fin de mejorar la ingesta de nutrientes de los consumidores o mejorar su salud en general (Nieto y Ros, 2012). La adición de ácidos grasos *n-3* y CLA en las dietas de los terneros puede ser una manera prometedora y sostenible para mejorar el valor nutricional de la carne, sin forzar a los consumidores a cambiar sus hábitos alimenticios.

Las decisiones de compra están basadas sobre una evaluación simultánea de múltiples atributos del producto, por lo que es importante conocer la respuesta de los consumidores hacia nuevos productos cárnicos. Existe una amplia información sobre los beneficios saludables, el papel biológico y el metabolismo de los ácidos grasos *n-3* y CLA, sin embargo, la información sobre la aceptación y actitud de los consumidores hacia carnes y productos cárnicos enriquecidos en estos ácidos grasos es limitada.

La deducción de los consumidores de la calidad del producto en el punto de compra se basa en las señales (*cues*) extrínsecas e intrínsecas disponibles. Es decir, el consumidor intenta relacionar las cualidades que tendrá la carne cuando la coma, con el juicio de valor que realiza en el momento de la compra de forma intuitiva o analítica, al tomar la decisión de elegir la carne por su apariencia. Estas señales que reflejan la calidad del producto pueden ser extrínsecas (precio, presentación del producto, origen y marca) e intrínsecas (características fisiológicas del producto tales como el color y la grasa visible) (Troy & Kerry, 2010). Comprender estas señales y cuáles son más importantes para el consumidor es esencial para que la industria cárnica pueda mejorar esos atributos en los productos nuevos y en los ya existentes.

El tipo de dieta de cebo de los terneros es un indicador de seguridad, de valor nutritivo y de calidad saludable de la carne para el consumidor español (Bernués *et al.*, 2003a). Asimismo, Olaizola *et al.* (2005) evaluaron la importancia de la calidad de la carne en España, e indicaron que el régimen de la alimentación animal, el origen / región, las condiciones de producción, bienestar animal, y la matanza fueron considerados los factores más importantes que influyen en la calidad de la carne por los consumidores. Cuando se comparan carnes procedentes de diferentes países, el país de origen, seguido por la alimentación animal y el precio fueron los factores más importantes que tienen en cuenta los consumidores españoles (Realini *et al.*, 2013).

Los atributos intrínsecos identificados por los consumidores son el color, como indicador de calidad y frescura (Faustman & Cassens, 1990; Glitsch, 2000), y la cantidad de grasa visible como indicador de salud (Issanchou, 1996) y palatabilidad (Sánchez *et al.*, 2012).

Aunque estudios previos han identificado indicadores de calidad en la carne de vacuno, hay que tener en cuenta que la percepción de la calidad de carne por parte del consumidor es compleja, difícil de definir y dinámica (Troy & Kerry, 2010). Los consumidores tienen distintas percepciones de lo que significa la calidad y por ello se pueden agrupar según sus preferencias y sus características socio-culturales. Por ejemplo, Sepúlveda *et al.* (2008) discriminaron el tipo de consumidor en España entre los no compradores y los compradores de carne que les interesaba la información en el momento de la compra. Bernués *et al.* (2003) en un estudio previo realizado en varios países europeos, entre ellos España, caracterizaron cuatro tipos de consumidores, entre ellos un tipo de consumidores que valoraba la trazabilidad y los controles de calidad del producto, así como la información nutritiva de producto, la marca, el origen, el tiempo de maduración y la fecha de caducidad o consumo preferente. Salud, satisfacción en el consumo y seguridad, fueron los aspectos más importantes de la actitud de consumidores irlandeses, mientras que precio y bienestar animal fueron los menos importantes. Además, la propaganda o información acerca de las cualidades nutritivas de la carne puede incrementar su consumo, en respuesta al mensaje de salud (McCarthy *et al.*, 2003). Beriaín *et al.* (2009) concluyeron que tanto los consumidores españoles como norteamericanos que son capaces de apreciar la calidad de la carne, están dispuestos a pagar más por un aumento de esa calidad.

Los determinantes de la predisposición a consumir carne certificada en España son el nivel de renta, el nivel de consumo, el precio medio y la percepción de seguridad (Angulo y Gil, 2007).

Las etiquetas o marcas en la carne son importantes, puesto que pueden servir al consumidor para valorar el producto y elegirlo en el momento de compra. En la comunidad europea el etiquetado de la carne de vacuno debe llevar una serie de indicaciones obligatorias entre las que destacan: un número de referencia o código de referencia, que garantice la relación entre la carne y el animal, el número de autorización del matadero y de la sala de despiece, país de nacimiento, engorde y sacrificio, todo ello para garantizar la trazabilidad, y está establecido en el Reglamento (CE) nº 1760/2000 (DOUE, 2000a) y las disposiciones de su aplicación en el Reglamento (CE) 1825/2000 (DOUE, 2000b). Además, existe un etiquetado facultativo que es una indicación adicional a las menciones obligatorias, que se recoge en un pliego de condiciones autorizado por la autoridad competente y certificado por un organismo independiente de control tal como establece el Real Decreto 1698/2003 de aplicación de los reglamentos comunitarios sobre sistemas de etiquetado de carne de vacuno (BOE, 2003). En base a este pliego facultativo se podrá utilizar las menciones facultativas en la etiqueta, donde se especifican las características específicas de calidad de esa carne (tipo de alimentación del animal, tiempo mínimo de maduración de la carne, tipo de grasa, etc.).

Se ha establecido la importancia de los ácidos grasos poliinsaturados (PUFA) de consumo para la salud humana. El mercado de ingredientes *n-3* creció un 24,3% el año pasado, lo que afirma su popularidad y la concienciación pública de sus beneficios (Ganesan *et al.*, 2014). No obstante, los consumidores no están dispuestos a renunciar al gusto de los alimentos funcionales por eventuales beneficios sobre la salud, por lo que el sabor se considera un factor extremadamente crítico para el futuro de la aceptación de los alimentos funcionales (Verbeke, 2006).

4. La carne en el ámbito de los alimentos saludables

4.1. Carne y derivados más saludables

El creciente interés por una alimentación saludable ha dado lugar a la aparición en el mercado de una nueva gama de alimentos y productos que, además de nutrir, mejoran la salud incrementando el bienestar y reduciendo el riesgo de contraer determinadas enfermedades. Estos alimentos se denominan genéricamente funcionales y, de acuerdo con los resultados de la acción concertada europea Functional Food Science in Europe (FUFOSE), se definen como "un alimento natural o uno al que se le ha añadido o eliminado componentes, por vía tecnológica o biotecnológica, de forma que se ha demostrado satisfactoriamente que tiene un efecto beneficioso para la salud además de los efectos nutricionales habituales" (EUR 1859, 2000).

La carne y productos cárnicos son elementos esenciales de la dieta humana que constituyen una importante fuente de proteínas, grasa, aminoácidos esenciales, minerales, vitaminas y otros nutrientes (Biesalski, 2005).

A medida que la economía se desarrolla, la carne y productos cárnicos no sólo se emplean para proporcionar los nutrientes necesarios, sino también se espera que tengan funciones adicionales para prevenir enfermedades y mejorar la salud mental y el bienestar de los consumidores (Roberfroid, 2000; Siro *et al.*, 2008). Estas demandas ofrecen grandes oportunidades para la industria de la carne. En la actualidad se ha multiplicado de forma importante la comercialización de estos alimentos funcionales en los países desarrollados.

A pesar de las numerosas modificaciones industriales llevadas a cabo en el desarrollo de alimentos funcionales, son pocas las pruebas científicas de que el consumo de un alimento genere un beneficio, identificado y definido, sobre la salud.

Todas las propiedades beneficiosas específicas de un alimento funcional, deben quedar reflejadas en el etiquetado y cumplir con lo establecido en el Reglamento 1924/2006 de la UE relativo a las alegaciones nutricionales y de propiedades saludables en los alimentos. Las alegaciones deberán ser ciertas, no

inducir a engaño y deben estar validadas científicamente. El citado reglamento se ha ido modificando por los siguientes: Reglamento (CE) nº 107/2008, Reglamento (CE) nº109/2008, Reglamento (CE) nº1169/2011 y Reglamento (CE) nº1047/2012.

La legislación debe ser totalmente estricta en lo referente a los mensajes y alegaciones de salud, donde es muy fácil confundir o no ser estrictamente honestos. Es muy importante que en el etiquetado de los productos se señale de forma clara cuáles son los posibles beneficios y riesgos de su consumo. Debe definir además cuál es la *población diana* de ese producto. El consumidor debe recibir junto con el cárnico funcional, una información correcta, adecuada y suficiente de lo que es y lo que contiene, muy en línea con el concepto de trazabilidad, así como de sus posibles efectos positivos para la salud. La instauración sólida de unas bases y control de estos nuevos alimentos constituyen un reto para la comunidad científica, las autoridades en el campo de la salud y la industria alimentaria.

4.2. Estrategias para la producción de carne y derivados más saludables

Apoyándose en la investigación científica se han acometido diferentes estrategias para el desarrollo de productos cárnicos más saludables que se recogen en dos grupos:

1. Genéticas o nutricionales. Nivel de producción animal.

2. Reformulación de productos cárnicos.

La composición de las canales y por tanto, también de los cortes comerciales, varía no sólo según la especie, sino también de la raza, edad, sexo, tipo de alimentación, etc. Una amplia gama de estrategias está disponible para inducir cambios en los diferentes componentes, tales como proteína, contenido lipídico, composición de ácidos grasos, nivel de vitamina E, etc. Estos incluyen selección genética, gestión de la nutrición y alimentación, agentes promotores del crecimiento,

inmunización de los animales y técnicas de manipulación genética (Bass *et al.*, 1990; Byers *et al.*, 1993; Hay y Preston, 1994).

La reformulación de productos cárnicos es la estrategia, al menos teóricamente, más simple y más utilizada en el desarrollo de nuevos productos funcionales. La modificación de la formulación se puede llevar a cabo de dos formas distintas:

- Reduciendo algunos compuestos normalmente presentes en estos alimentos a cantidades apropiadas, como la grasa, AGS, colesterol, sal, nitritos, etc.
- Incorporando ingredientes funcionales, por ejemplo, fibras, ciertos tipos de proteínas vegetales, AGM y AGPI, antioxidantes, etc.

Existen numerosos aspectos que deben tenerse en cuenta en el desarrollo de este tipo de productos (Jiménez-Colmenero, 2000). Pueden aparecer problemas derivados de las posibles interacciones entre los ingredientes funcionales y los componentes de la matriz alimentaria. El efecto de estas interacciones se puede traducir, en el aspecto nutricional, en una disminución de la biodisponibilidad del principio activo adicionado o en la variación de las condiciones óptimas para su absorción y respecto a su calidad, en una alteración de las características sensoriales del producto final que provoque el rechazo del mismo por el consumidor.

Doce grandes grupos de componentes (de origen animal y vegetal) han sido identificados como poseedores de efectos potencialmente beneficiosos para la salud humana (Goldberg, 1994): fibra dietética; oligosacáridos; azúcares/alcoholes; aminoácidos, péptidos y proteínas; glucósidos; alcoholes; isoprenos y vitaminas; colina; bacterias del ácido láctico; minerales; ácidos grasos insaturados; y otros no incluidos en las categorías anteriores como, por ejemplo, los antioxidantes.

4.3. Empleo de extractos de semilla de uva y aceite de oliva en derivados cárnicos

4.3.1. Extractos de semilla de uva

El extracto de semilla de uva (ESU) es un extracto rico en compuestos polifenólicos que se obtiene a partir de subproductos de vinificación. El ESU se vende comercialmente como suplemento dietético y aparece listado en el EAFUS (Everything Added to Food in the United States) y se ha reconocido como una sustancia segura (GRAS) según la Administración de alimentos y drogas (FDA).

Composición general de la uva

Los compuestos polifenólicos de la uva se distribuyen en el jugo (10%), en la piel (30%) y en las semillas (60%) (Vinson y Hontz, 1995) y en función de su importancia cuantitativa, se encuentran: los taninos, los antocianos, los ácidos fenólicos, los flavonoles y flavanonoles y los estilbenos. En la figura 10 se puede observar la distribución de estos polifenoles en los granos de uva.

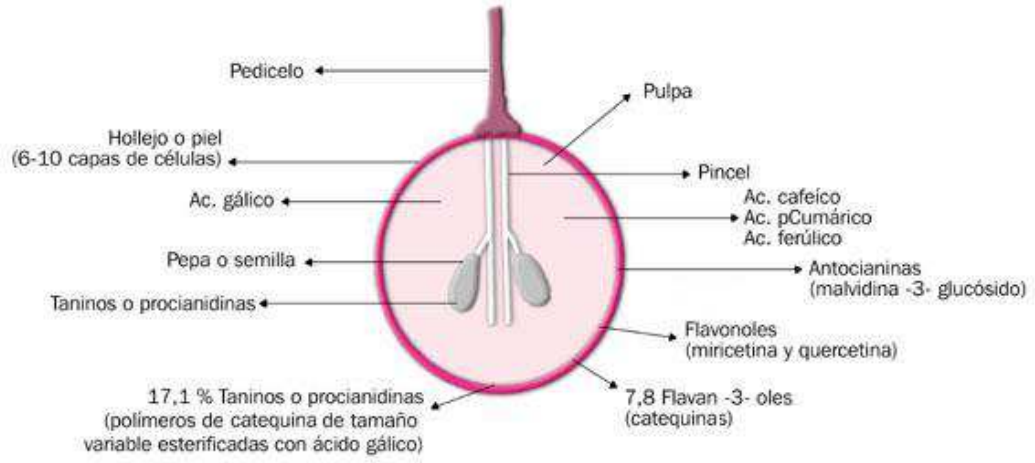


Figura 10. Distribución esquemática de los polifenoles en un grano de uva tinta de *Vitis vinífera*, variedad Cabernet Sauvignon.

La concentración y variedad de polifenoles en la uva depende de numerosos factores, tales como la variedad de vid, clima, terreno y cosecha temprana o tardía

(Infante, 1997). La tabla 3 recoge los contenidos medios de los principales compuestos en las bayas (granos) de *Vitis vinifera*.

Tabla x. Contenido medio de compuestos fenólicos en las bayas de las variedades de *Vitis vinifera* más cultivadas (según J.M. Souquet *et al.*, 1996) (expresado en mg/kg de bayas)

Compuestos fenólicos	Pulpa	Hollejos	Pepitas
Taninos	Trazas	100 a 500	1000 a 6000
Antocianos	0 (1)	500 a 3000 (2)	0
Ácidos fenólicos	20 a 170	50 a 200	0

(1) Excepto variedades tintoreras.

(2) Contenido en variedades tintas.

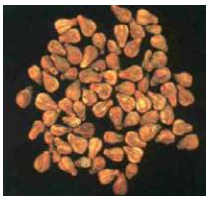
Los antocianos se encuentran fundamentalmente en el jugo vacuolar de las células del hollejo y pueden excepcionalmente encontrarse en la pulpa de variedades tintoreras. Los ácidos fenólicos se localizan en las vacuolas de las células tanto de la pulpa como del hollejo. Los flavonoles están presentes únicamente en los hollejos de la uva tanto blanca como tinta, y los flavanonoles se han identificado en hollejos de uvas blancas. Los estilbenos sólo están presentes en los hollejos donde es sintetizado, y en contenidos variables, en función de las variedades.

Los taninos, tanto monómeros como en formas más o menos polimerizadas, se localizan principalmente en las semillas, aunque se han localizado también trazas de monómeros y dímeros en la pulpa (Bourzteix *et al.*, 1986; Ricardo da Silva *et al.*, 1992). Otra fuente importante de taninos es el hollejo de las uvas, donde se han identificado tres tipos de taninos. Los contenidos de taninos de las semillas son siempre netamente superiores a los del hollejo, ya sea de monómeros, oligómeros o polímeros, tal y como se puede observar en la tabla 4. Los taninos de los hollejos difieren de los de las semillas por la presencia de prodelfinidinas, mayor grado de polimerización (Di Stefano, 1995) y menor porcentaje de subunidades galoiladas.

Tabla x. Comparación de los contenidos en catequina, procianidinas dímeros y trímeros de las pepitas y hollejos de las variedades Merlot y Cabernet Sauvignon en el momento de la madurez en 1994 (según Víctor de Freitas, 1995)

Variedades		Catequina	Procianidinas dímeros	Procianidinas trímeros
Merlot	Pepitas	146	291	33
	Hollejos	7	11	3
Cabernet Sauvignon	Pepitas	210	273	63
	Hollejos	4	11	1

Polifenoles en el extracto de semilla de uva

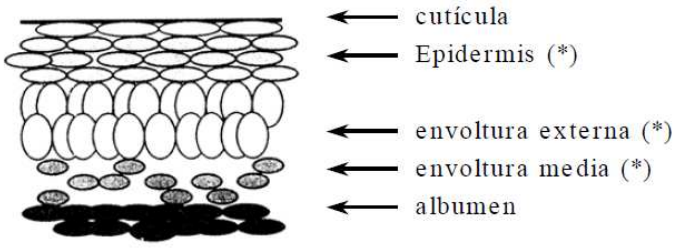


Las semillas de uva están compuestas por 7% de fenoles, 40% de fibra, 16% de aceite, 11% de proteínas, azúcares y sales minerales (Murga *et al*, 2000).

Figura 11. Semillas de uva.

Los polifenoles presentes en las semillas son los taninos, que son más abundantes en las semillas de las variedades tintas que las semillas de las variedades blancas (Fuleki y da Silva, 1997).

Los taninos de la semilla se encuentran localizados en las capas superficiales de la misma, ocupando una posición de defensa del embrión en las envolturas externas e internas (Thorngate y Singleton, 1994).



(*) Presencia de taninos (reacción al $FeCl_3$)

Figura 12. Localización de taninos en la semilla de uva (Saint-Cricq *et al.*, 1999).

La estructura de los taninos en las semillas de uva se basa en polímeros más o menos complejos formados por unidades de flava*n*-*3*-oles o 3-flavanoles. Los flava*n*-

3-oles, comúnmente llamados catequinas poseen dos ciclos bencénicos unidos por un heterociclo oxigenado saturado (núcleo fenil-2 cromano). Esta estructura presenta dos carbonos asimétricos (C2 y C3) que originan cuatro isómeros (Figura 13). Los principales taninos catequinos monómeros de la baya de uva son la (+)–catequina, la (-)epicatequina, la galocatequina y la epigalocatequina (Souquet *et al.*, 1996a, Cheynier *et al.*, 1998).

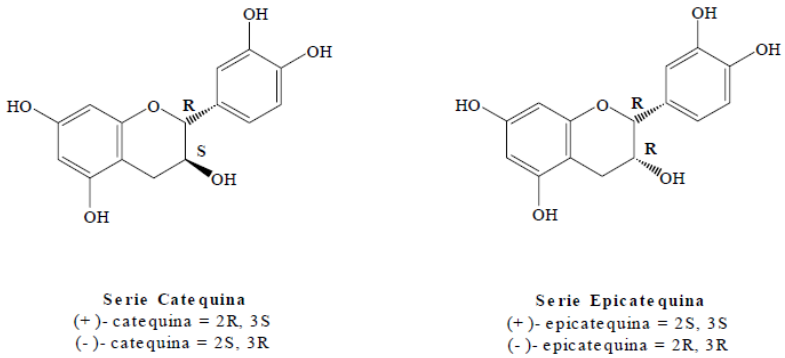


Figura 14. Estructura de los flava*n-3*-oles, unidades básicas de las procianidinas.

Los oligómeros y polímeros de los flava*n-3*-oles se conocen también con el término de proantocianidinas (o proantocianidoles), que proviene de su propiedad de liberar antocianidoles (o antocianas), en medio ácido y caliente, por ruptura de las uniones intermonoméricas (Porter *et al.*, 1985; Souquet *et al.*, 1996; Cheynier *et al.*, 1998). Dentro de las proantocianidinas, reciben el nombre de procianidinas aquellas que derivan de la catequina y la epicatequina, que en hidrólisis ácida generan la molécula de cianidina; y el nombre de prodelfinidinas a aquellas derivadas de la galocatequina y la epigalocatequina, que se hidrolizan en delfinidina en medio ácido.

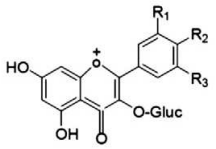
Las estructuras básicas con grupos 3, 4, 5-trihidroxifenil encontradas en epigalocatequina, epigalocatequina-3-Ogalato, castalagina y prodelfinidina pudieran ser importantes para la actividad antibacteriana (Tagurt *et al.*, 2004). Anastasiadi *et al.* (2009) sugirieron que altas concentraciones de flavonoides y sus derivados en las semillas de uvas eran responsables de la actividad antimicrobiana.

Las procianidinas dímeras son las más simples y tienen uniones de tipo C4–C8 entre los monómeros. Los dímeros procianidínicos más comunes son B1, B2, B3

y B4. Estos están seguidos de los isómeros con uniones C4–C6, tales como B5, B6, B7 y B8 (Castillo *et al.*, 2000; Yilmaz y Toledo, 2004).

Las procianidinas oligoméricas corresponden a polímeros formados por un número amplio de unidades flavanoles (de tres a una decena). Las formas poliméricas representan más del 55% de los 3-flavonoles en semillas de uva (Saito *et al.*, 1998; Kennedy *et al.*, 2000; Jayaprakasha *et al.*, 2001; Yilmaz *et al.*, 2004) y se localizan en su mayoría en la cubierta marrón de la semilla (Thorngate y Singleton, 1994).

Las procianidinas se pueden combinar con ácido gálico para formar ésteres de galato y finalmente glucósidos (Figura 15) (Negro *et al.*, 2003; Weber *et al.*, 2007). El color rojo y sabor astringente del ESU puede ser atribuido a los polifenoles, especialmente a las proantocianidinas, que pueden afectar el color y las características sensoriales del producto cuando se usan en concentraciones más altas (Monteleone *et al.*, 2004; Weber *et al.*, 2007).



anthocyanins

	R_1	R_2	R_3
peonidin-3-*O*-glucoside	OCH_3	OH	-
petunidin-3-*O*-glucoside	OH	OH	OCH_3
malvidin-3-*O*-glucoside	OCH_3	OH	OCH_3
cyaniding-3-*O*-glucoside	OH	OH	-
delphinidin-3-*O*-glucoside	OH	OH	OH

Figura 16. Estructuras químicas de algunos compuestos fenólicos de semillas de uva.

El grado de polimerización de las procianidinas puede determinar la actividad antioxidante ya que a más alto grado de polimerización, mayor actividad antioxidante (Spranger *et al.*, 2008).

Según diversos estudios (Jacob *et al.*, 2008; Sato *et al.*, 1996; Soobrattee *et al.*, 2005), las propiedades antioxidantes del ESU son principalmente debidas a los flavonoides que pueden secuestrar radicales libres, quelar metales, reducir la formación de hidroperóxidos y presentar efectos de señalización celular y expresión génica. El potencial antioxidante de ESU es veinte y cincuenta veces superior a los de las vitaminas E y C, respectivamente (Shi *et al.*, 2003).

Efectos de los extractos de semillas de uva en productos cárnicos

Existen diversos estudios en los que se ha aplicado el ESU sólo o en combinación con otras tecnologías en distintas carnes crudas y cocinadas (Ahn *et al.*, 2002; Ahn *et al.*, 2007; Bañón *et al.*, 2007; Brannan, 2009; Brannan y Mah, 2007; Carpenter *et al.*, 2007; Din Shirahigue *et al.*, 2010; Gadang *et al.*, 2008; Kulkarni *et al.*, 2011; Lau y King, 2003; Mielnik *et al.*, 2006; Nissen *et al.*, 2004; Özvural y Vural, 2011; Rojas y Brewer, 2007; Rojas y Brewer, 2008; Selani *et al.*, 2011).

En los sistemas cárnicos, el ESU muestra la actividad antioxidante reduciendo la cantidad de productos primarios de la oxidación de lípidos (por ejemplo, hidroperóxidos lipídicos y hexanal) y productos secundarios de oxidación de los lípidos (por ejemplo, sustancias reactivas al ácido tiobarbitúrico – TBARS) (Brannan y Mah, 2007). El ESU ha reducido el desarrollo del flavor rancio y actividad antioxidante en diversos productos cárnicos como carne de vacuno cruda y cocinada (Ahn *et al.*, 2002; Bañón *et al.*, 2007), hamburguesas de carne de cerdo crudas y cocinadas (Carpenter *et al.*, 2007; Nissen *et al.*, 2004), pavo (Mielnik *et al.*, 2006), aceite de pescado y pescado congelado (Pazos *et al.*, 2004) y pechugas y muslos de pollo (Brannan y Mah, 2007; Brannan, 2009; Lau y King, 2003).

El nivel de concentración mínimo de ESU requerido para producir un efecto antioxidante en carne de cerdo cocinada fue de 400 µg/g y en pollo picado de 0,1% (w/w), reduciendo así el TBARS (Lau y King, 2003). La actividad antioxidante del ESU es dependiente de la concentración entre 0,02% y 0,1% (Ahn *et al.*, 2002). El extracto de semilla de uva al 0,1% (w/w) es un secuestrador eficaz de radicales en tejidos musculares y reduce los productos secundarios de la oxidación de en carne de vacuno, pollo y pavo durante el almacenamiento refrigerado (Ahn *et al.*, 2002; Mielnik *et al.*, 2006; Rababah *et al.*, 2006). La concentración de 0,1% w/w de ESU se puede utilizar para conseguir un efecto antioxidante eficaz tanto en los sistemas de carne cruda como cocinada. Además, el ESU (6000 ppm) no cambia las puntuaciones del flavor en pechugas de pollo irradiadas y no irradiadas (Rababah *et al.*, 2005). Por otra parte, no hubo efecto del ESU (0,1% w/w) sobre el pH, el rendimiento y actividad de agua en las muestras de pechuga de pollo picada (Brannan, 2009). Sin embargo, el ESU en concentraciones del 0,1% (w/w) no es un

antioxidante eficaz contra el flavor recalentado (WOF) en la carne de los muslos de pollo.

Las propiedades antimicrobianas del ESU han sido evaluadas en relación con *L. monocytogenes*, *S. Typhimurium*, *S. aureus*, *B. cereus*, *E. sakazakii*, *E. coli O157:H7*, *A. hydrophila*, y otros patógenos transmitidos por los alimentos, tanto *in vitro* como en los sistemas alimentarios (Ahn *et al.*, 2004; Anastasiadi *et al.*, 2009; Kim, *et al.*, 2005; Sivarooban *et al.*, 2007). Compuestos fenólicos extraídos de semillas de uva demostraron efectos inhibitorios sobre el *S. aureus* y *E. coli* (Rotava *et al.*, 2009).

El extracto de semilla de uva (1% w/w) demostró actividad antimicrobiana frente a bacterias Gram negativas como *E. coli O157:H7* y *S. Typhimurium* en carne picada y cocinada (Ahn *et al.*, 2007).

Para mejorar la seguridad y calidad de los alimentos en la industria agroalimentaria se está aplicando la tecnología de barreras u obstáculos (Juneja *et al.*, 2010) que permite mejoras en la seguridad y calidad de los alimentos, combinando varios factores (u obstáculos) como el envasado al vacío, el pH, la temperatura de almacenamiento, otros antimicrobianos como los tratamientos ácidos orgánicos (lactato, acetatos y diacetatos), tratamientos de calor post–proceso y la adición de extractos naturales de plantas como los ESU.

El ESU (1%) combinado con nisina (6400 UI/ml) inhibe las poblaciones de *L. monocytogenes* a niveles no detectables (límite mínimo de detección fue de 100 UFC/g) en salchichas de pavo conservadas a 4 °C y 10 °C (Sivarooban *et al.*, 2007). Por otra parte, las semillas de uva y extractos de té verde aumentaron la vida útil de hamburguesas crudas y de otros productos cárnicos almacenados en condiciones de venta al consumidor (Bañón *et al.*, 2007).

La liberación de los componentes fenólicos a través de tecnologías avanzadas, como la pulverización electrostática y el envasado de nanopartículas de estos compuestos activos en los alimentos, pueden proporcionar resultados prometedores y requieren una investigación extensa para utilizar estos extractos de plantas baratos, naturales y seguros (Ganesh *et al.*, 2010; Ravichandran *et al.*, 2010).

Son necesarias investigaciones adicionales para validar los efectos beneficiosos y la seguridad del uso de ESU. Hay que considerar el nivel muy bajo de concentración de ESU (0,01 a 1%) en aplicaciones alimentarias en comparación con las dosis farmacológicas (150-300 mg/kg) necesarias para conseguir los efectos beneficiosos en los seres humanos y animales (Clouatre y Kandaswami, 2005). No obstante, hay que indicar que se ha determinado un nivel de efecto adverso del ESU en ratas cuando se les administra en dosis de 1,78 g/kg de peso corporal/día, que es evidentemente un nivel de concentración mucho más alto de lo que normalmente se utiliza en aplicaciones alimentarias (Bentivegna y Whitney, 2002).

4.3.2. Aceite de oliva

Dentro de las estrategias de reformulación para la obtención de productos cárnicos más saludables se encuentra la mejora en el perfil lipídico basada en la sustitución en mayor o menor medida de la grasa animal normalmente presente en el producto, por otra cuyas características estén más en consonancia con las recomendaciones nutricionales. Una extensa variedad de grasas de origen vegetal (oliva, canola, girasol, etc.) y marino (algas y pescado) han sido utilizadas como sustitutos parciales de la grasa animal (principalmente cerdo y vacuno) en diversos productos cárnicos (Jiménez-Colmenero, 2007). Para una consecución adecuada de esta reformulación con aceites, es necesario que los lípidos incorporados queden bien integrados en el producto y que no supongan un deterioro en la percepción organoléptica del mismo.

Se han empleado tanto grasas en estado sólido como en estado líquido (Jiménez-Colmenero, 2007) y su incorporación ha dependido fundamentalmente del producto al que han sido incorporados. En tal sentido, se han ensayado cuatro maneras diferentes de incorporar aceites de origen vegetal y marino en la reformulación de productos cárnicos: adición de manera directa ya sea líquida o sólida (incluyendo interesterificación), adición como aceites encapsulados y adición como aceites emulsionados.

La incorporación de grasas en estado líquido conlleva una problemática derivada de la posible pérdida del aceite durante el procesado y/o cocinado, provocando una pérdida considerable en la calidad del producto final. Mediante

microinyección se ha incorporado aceite de oliva a productos cárnicos cocidos picados (Chatzigeorgiou, 2008; Domazakies, 2005). También se ha estudiado en productos tipo hamburguesa la incorporación directa de aceites (Dzudie *et al.*, 2004; Lowder & Osburn, 2010; Rodríguez-Carpena *et al.*, 2011).

Algunas de las grasas vegetales tienen consistencia sólida a temperatura ambiente debido a su alto contenido en glicéridos. El problema del procesado de grasas con altos puntos de fusión es que pueden producirse emulsiones con baja estabilidad debido a la mayor dificultad de picado y dispersión de la grasa (Whiting, 1987).

La microencapsulación de aceites hace posible la adición de ciertos tipos de aceites a diferentes alimentos y permite retrasar o incluso inhibir la oxidación lipídica (Kolanowski, Swiderski, & Berger, 1999). El uso de este tipo de tecnología es muy limitado en los productos cárnicos, aunque se han utilizado aceite de pescado y linaza encapsulados como sustitutos de la grasa animal en embutidos curados (Josquin, *et al.*, 2012; Pelser *et al.*, 2007).

A través de la adición del aceite en forma emulsionada, el aceite queda estabilizado en la matriz proteica y se dispersa mejor en alimentos acuosos (como los productos cárnicos), manteniéndose intactos sus sistemas antioxidantes y reduciéndose así las posibilidades de que el aceite se separe de la estructura del producto cárnico durante los periodos de procesado, conservación y consumo del mismo (Djordjevic *et al.*, 2004).

Dentro de todos los aceites empleados en los derivados cárnicos, el aceite de oliva es el lípido vegetal que ha recibido mayor atención por su alto valor biológico, principalmente como un aceite insaturado rico en antioxidantes naturales y una excelente fuente de ácidos grasos poliinsaturados (Jiménez *et al.*, 2010; Fernández *et al.*, 2009).

El aceite de oliva se extrae del fruto del olivo (Oliva europaea) denominado oliva o aceituna, mediante prensado de la drupa y posterior centrifugado y percolación. La composición del aceite de oliva se divide en componentes mayoritarios y minoritarios. Los mayoritarios suponen más del 98% del peso total y son fundamentalmente triacilgliceroles, mientras que los minoritarios son más de

230 compuestos químicos, muchos de los cuales poseen capacidad antioxidante (Sánchez-Muniz, 2007). Cabe destacar su abundancia en el ácido oleico, constituyendo entre el 60-85% del total de ácidos grasos de los triglicéridos; mientras que el ácido linoleico se halla en concentraciones del 3-21% (Serra-Majem *et al.*, 2003; Perona *et al.*, 2006).

Tanto el ácido oleico, como algunos polifenoles (tocoferol, hidroxitorisol y oleuropeína), son los componentes del aceite de oliva que pueden ejercer mayores beneficios sobre la salud humana (Huang & Sumpio, 2008; Sánchez-Muniz, 2007). Se ha constatado que el consumo de aceite de oliva produce una disminución del LDL colesterol cuando se hace en sustitución de AGS (por la alta cantidad de AGM), aporta gran cantidad de antioxidantes y puede reducir el desarrollo de aterosclerosis (Ruiz-Canela & Martínez-González, 2011). Otros estudios proponen que su consumo incrementa el HDL colesterol y la sensibilidad a la insulina y disminuye el daño oxidativo de lípidos y ADN (Covas, Konstantinidou, & Fito, 2009).

El tocino es la principal fuente de grasa animal en algunos productos cárnicos y su sustitución por aceite de oliva en forma de emulsión puede mejorar el perfil lipídico, por la disminución de AGS y el aumento de ácidos grasos insaturados, en especial AGM. Por ello, varios autores han empleado aceite de oliva emulsionado en el desarrollo de productos cárnicos tipo embutidos crudo curados o salchichas con el fin de reducir su contenido en grasa (Ansorena & Astiasarán, 2004a, 2004b; Beriain *et al.*, 2011; Bloukas *et al.*, 1997; Muguerza *et al.*, 2002; Muguerza *et al.*, 2001; Severino *et al.*, 2003). También se ha adicionado aceite de oliva en forma de emulsión en hamburguesas de vacuno obteniendo resultados satisfactorios desde el punto de vista sensorial y nutricional (López-López *et al.*, 2010, 2011; Martínez *et al.*, 2012).

2. Objetivos y Diseño experimental

1. Objetivos

El **objetivo general** planteado en la presente Tesis Doctoral es:

Estudiar el efecto de la incorporación de ingredientes ricos en ácidos grasos poliinsaturados *n-3* y CLA en las dietas de cebo de terneros con objeto de mejorar la calidad nutricional y organoléptica de su carne, en función de las preferencias de los consumidores nacionales, y posibilitar la obtención de nuevos derivados cárnicos saludables.

Dicho **objetivo general** se desglosó en los siguientes **objetivos específicos**:

1. Evaluar el efecto de la utilización de dietas enriquecidas con ácidos grasos *n-3* y/o CLA sobre los parámetros productivos, la calidad de la canal y el metabolismo de los lípidos en terneros.

2. Estudiar el perfil de ácidos grasos de la grasa intramuscular y subcutánea de la carne de ternera enriquecida en ácidos grasos *n-3* y/o CLA, y su adecuación a las recomendaciones nutricionales de la composición de la grasa en la dieta humana.

3. Estudiar la aceptabilidad de la carne de ternera enriquecida en *n-3* y/o CLA por los consumidores, así como la actitud de éstos frente a los alimentos funcionales, teniendo en cuenta los factores socioeconómicos que determinan la respuesta de los mismos.

4. Examinar la vida útil y estabilidad oxidativa en carne picada y uso del ESU como antioxidante natural para inhibir la oxidación lipídica que puede tener lugar en este tipo de carnes.

5. Examinar la vida útil y estabilidad oxidativa en hamburguesas bajas en grasa y elaboradas con aceite de oliva emulsionado y uso del ESU como antioxidante natural.

2. Diseño experimental

El diseño experimental llevado a cabo en la presente Tesis Doctoral, queda representado el la figura 17.

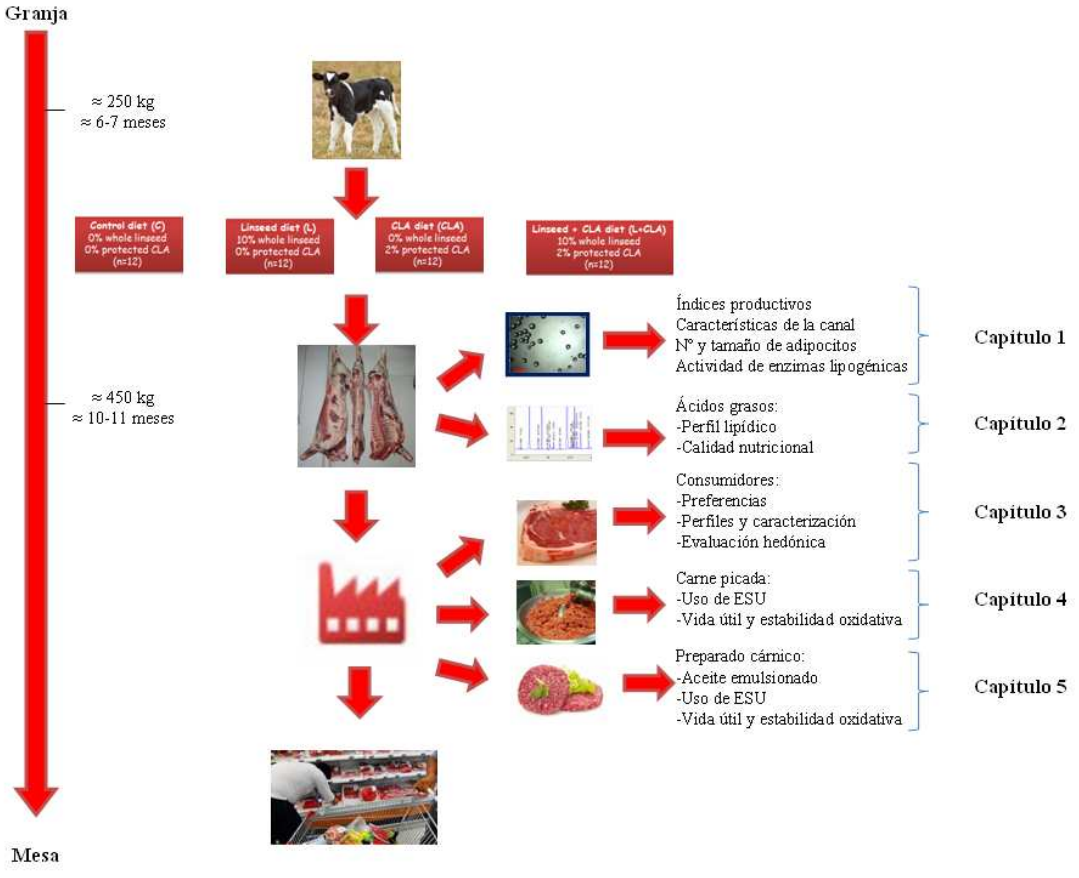


Figura 17. Diseño experimental.

3. Características de la canal bovina y desarrollo del tejido adiposo

Effect of linseed and/or conjugated linoleic acid supplementation on performance and adipose tissue development in young Holstein bulls

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Abstract

The object of this experiment was to study the effect of whole linseed and conjugated linoleic acid (CLA) supplementation of concentrate on performance, carcass characteristics, and adipose tissue development in young bulls. Forty-eight young Holstein bulls were distributed into four diet groups: *Control* [C] (0 % linseed, 0 % conjugated linoleic acid (CLA); n=12), *Linseed* [L] (10 % linseed, 0 % CLA; n=12), *CLA* [CLA] (0 % linseed, 2 % CLA; n=12) and *Linseed + CLA* [L+CLA] (10 % linseed and 2 % CLA, n=12). The four diets were isoenergetic (3.34 Mcal ME·kg^{-1}) and isoproteic (16.9 % CP). Animals were fattened from 239.8±6.61 to 458.6±9.79 kg body weight (322±6.0 d old at slaughter). Average weight gain of the bulls was high, more than 1.7 kg/d, but performance and carcass classification scores were unaffected by diet composition. Adding linseed or CLA to the concentrate diet did not result in significant differences in adipocyte size or lipogenic enzyme activity. However, while the frequency distribution of subcutaneous adipocyte diameters followed a normal distribution, the frequency distribution of intramuscular adipocyte diameters was not normal in any of the four dietary groups (skewness coefficients: 0.8, 1.2, 0.9, 0.8 for C, L, CLA, and L+CLA, respectively; $P < 0.05$), indicative of possible adipocyte proliferation in this latter adipose tissue. In conclusion, the findings reported here showed that adding 10 % linseed and/or 2 % CLA to an isoenergetic, isoproteic concentrate diet for fattening young bulls did not bring about any significant differences in performance, carcass characteristics, or adipose tissue development in the test animals.

Key words: Linseed, Conjugated linoleic acid, Adipocytes, Lipogenic enzyme activity, Young bulls

1. Introduction

Beef is perceived by some consumers as unhealthy because of its high saturated fat content and its purported relationship to higher rates of certain diseases (Ferguson, 2010). Saturated fats are known to raise blood cholesterol levels and heighten the risk of cardiovascular disease.

The fatty acid profile of the intramuscular fat of beef can be altered by including ingredients rich in polyunsaturated fatty acids in the fattening diets administered to the animals (Wood *et al.*, 2004). Different polyunsaturated fatty acid-rich ingredients are being tested for this purpose, though biohydrogenation of polyunsaturated fatty acids in the rumen and liver metabolism limits their availability to be metabolized and to accumulate in the fat depots (Gruffat, Gobert, Durand & Bauchart, 2011).

Linseed is a ready, natural source of linolenic fatty acid, and its seed coat may afford PUFAs some protection against rumen biohydrogenation and thus increase the passage of PUFAs into the duodenum. It is also a precursor of eicosanoids, which play an important antithrombotic and anti-inflammatory role (Palmquist, 2009).

Conjugated linoleic acid (CLA) comprises a group of isomers of linoleic acid having a number of biological activities. The cis-9, trans-11 isomer appears to be active in inhibiting carcinogenesis in animal models (Pariza, Park & Cook, 2001), whereas the trans-10, cis-12 isomer affects lipid metabolism (Pariza, 2004). Dietary CLA has been reported to reduce body fat in several species (Azain, 2003; Ostrowska, Muralitharan, Cross, Bauman & Dunshea, 1999). In pigs, CLA reduces backfat and increases marbling (Barnes, Winslow, Shelton, Hlusko & Azain, 2012). In cattle, this effect on the amount of fat deposited in the various depots remains nuclear at the present time (Schiavon et al., 2011).

The effect of the intake of polyunsaturated fatty acid-rich ingredients like linseed and CLA on adipocyte hypertrophy and hyperplasia and on the activity of

lipogenic enzymes is also not precisely understood. Similarly, data on growth rate and feed efficiency for different species are conflicting (Belury, 2002; Chin, Liu, Storkson, Ha & Pariza, 1992; Thiel-Cooper, Parrish, Sparks, Wiegand & Ewan, 2001).

Accordingly, the object of this study was to examine the effects of feeding a concentrate diet including whole linseed, rich in n-3 fatty acids, and/or protected conjugated linoleic acid (CLA), rich in n-6 fatty acids, on performance and adipose tissue development and metabolism in young Holstein bulls.

2. Materials and methods

2.1. Animals and feeding

Forty-eight Holstein calves were divided into four sample groups of twelve animals each, logged at the CITA experimental farm in Aragón (Spain). Each group of animals was fattened using one of four concentrate diets tested (composition and chemical analysis detailed in Table 1). All four diets were formulated to be isoenergetic and isoproteic and had similar ether extract (7 %) and starch (35 %) contents, differing in the percentage of added linseed and/or CLA (Lutrell® pure, BASF, Germany): control (0 % linseed, 0 % CLA), linseed (10 % linseed, 0 % CLA), CLA (0 % linseed, 2 % CLA) and linseed + CLA (10 % linseed and 2 % CLA) [respectively abbreviated C, L, CLA, and L+CLA]. Whole linseed was added to the ground concentrate. The CLA supplement consisted of methyl esters of CLA bound to a colloidal silica matrix and coated with hydrogenated vegetable fats to protect from ruminal degradation. The vitamin E content of all the diets was 110 mg vitamin E / kg.

The animals were weighed at the start and end of the experiment and every two weeks, all at the same time. Concentrate or admixture intake for each group was measured monthly, and feed efficiency in relation to weight gain was calculated.

Care and use of the animals was in accordance with European guidelines (EU, 2010).

2.2. Slaughter and sample collection

Slaughter weight was set at 450 kg. Animals were transported 10 km and slaughtered at the Mercazaragoza abattoir. Hot carcass weight at slaughter was recorded, and a coefficient of 0.98 was applied to convert to cold carcass weight. Dressing percentage was calculated as the ratio of cold carcass weight to final live weight (LW). Carcasses were graded for conformation and fatness according to EU Regulations Nos. 1208/81 and 1026/91 (EEC, 1991). SEUROP conformation was converted to an 18-point scale, scoring 1 for P- to 18 for S+, and fatness 1 to 5 was converted to a 15-point scale, scoring 1 for 1- to 15 for 5+.

Fat samples were taken immediately after slaughter. An amount of 10 g of subcutaneous (SC; right-side 10th rib) and an amount of 10 g of intramuscular (IM; *Longissimus dorsi* muscle) fat were collected and used to determine adipocyte size and lipogenic enzyme activity. The samples for the adipocyte size determination (2 g) were stored in glass vials with 10 mL of Tyrode's solution (0.15 M NaCl; 6 mM KCl; 2 mM $CaCl_2$; 6 mM glucose; 2 mM $NaHCO_3$; pH 7.62) at a temperature of 39 ºC. The samples for the enzyme activity determination (5 g) were frozen in liquid

nitrogen and stored at -80 ºC until analysis. The remaining tissue was frozen and used to determine the lipid content (Soxhlet method, ISO 1443-1973).

Twenty-four hours after slaughter the left-side 10th rib was removed, weighed, and stored at 4 °C for later dissection to determine the amount of subcutaneous and intramuscular fat of the piece. Tenth rib tissue composition is considered representative of the tissue composition of the whole carcass (Oliván, Martínez, García, Noval & Osoro, 2001). In addition, the marbling area and number of marbling flecks were determined by computerized image analysis for the *Longissimus dorsi* muscle (LM) removed from that same rib (Mendizabal, Purroy, Indurain & Insausti, 2005).

2.3. Adipocyte size

The samples used to determine adipocyte size were digested with collagenase to dissolve the matrix of connective tissue surrounding the adipocytes (Rodbell, 1964). On arrival at the laboratory, the samples preserved in Tyrode's solution were immediately removed from the collection vial and cut into smaller pieces to augment the surface area exposed to the collagenase. Enzyme digestion took place in a Petri dish in an incubator to maintain the temperature of the fat sample. The samples mixed with collagenase (5 ml M199 media, 200 mg bovine seroalbumin, and 5 mg collagenase) were held at 39 °C for 2 hours. After digestion the samples were removed from the incubator and filtered to separate the fatty tissue, which had not been digested by the collagenase. Slides of suspended adipocytes were prepared for examination under the microscope. The microscope images obtained were digitized

and saved on a computer storage medium for measurement of adipose cell diameter (180-200 adipocytes per depot per animal) using image analysis software (Image-Pro Plus version 5.1).

2.4. Enzyme assays

The following lipogenic enzymes were assayed: glycerol 3-phosphate dehydrogenase (G3PDH; EC 1.1.1.8) (Wise & Green, 1979), an enzyme involved in glycerol 3-phosphate synthesis from glucose; fatty acid synthase (FAS; EC 2.3.1.85) (Halestrap & Denton, 1973), involved in *de novo* fatty acid synthesis; glucose 6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49) (Glock & McLean, 1953); and reduced nicotinamide adenine dinucleotide phosphate (NADPH)-isocitrate dehydrogenase (ICDH; EC 1.1.1.50) (Plaut, 1962), these two last-mentioned enzymes involved in reduced nicotinamide adenine dinucleotide phosphate supply for *de novo* fatty acid synthesis.

The enzyme activity assays were carried out using tissue homogenates prepared from the fat samples (5 g) which had been stored frozen (-80 °C), and enzyme activity was determined using a spectrophotometer. Tissue homogenates were prepared in ice cold STEG buffer pH 7.4 (0.3M saccharose, 30mM Trizma® base, 1mM EDTA, 1mM glutathione) (1:4 w/v) using a Sorvall Omni-Mixer homogenizer (50 000 rpm/10 s 3 times, with 10-s pauses between grinding to prevent sample warming). Homogenates were filtered (20-µm pore diameter filter) and centrifuged (6 000 rpm/4 °C/10 m). Supernatants were filtered (20-µm pore diameter filter) and centrifuged (11 000 rpm/4 °C/10 m). The second supernatants were filtered (0.45-µm pore diameter filter) and used for the enzyme assays. Enzyme activities were determined by measuring

variations in absorbance over time at 37 °C. Reactions were linear with respect to time over the period of the assay and proportional to the amount of extract added. To calculate enzyme activity per 10^6 cells, the number of cells per gram of adipose tissue was calculated using mean adipocyte volume, the lipid content of the tissue (determined by the Soxhlet method according to ISO 1443-1973), and lipid density (0.915 g/mL).

2.5. Statistical analysis

Statistical analyses were performed using SAS v. 9.1 (SAS, 2011). Weight, average daily gain, dressing percentage, carcass classification score, percentage rib composition, *Longissimus dorsi* muscle and marbling area, fat fleck size and number, adipocyte size, and lipogenic enzyme activity were tested by analysis of variance using the GLM procedure. Significant differences between means were identified applying Tukey's test using the following equation:

$$y_{ij} = \mu + C_i + e_{ij}$$

where y_{ij} = daily gain, dressing percentage, carcass classification score, percentage rib composition, *Longissimus dorsi* muscle area and fat fleck size and number, adipocyte size, or lipogenic enzyme activity; μ = least squares mean value, C_i = fixed effect of concentrate diet type (i = 1: control (0 % linseed, 0 % CLA); i = 2: linseed (10 % linseed, 0 % CLA); i = 3: CLA (0 % linseed, 2 % CLA); i = 4: linseed + CLA (10 % linseed and 2 % CLA); e_{ij} = random residual effect.

3. Results and discussion

3.1. Performance

The mean initial weight of the young bulls was about 239.8 ± 22.12 kg (Table 2). As slaughter weight was set at 450 kg, there were no statistical differences in final weight between the groups, which varied from 450.5 kg LW (control group) to 468.8 kg (L+CLA group). Mean age at slaughter was about 11 months, hence the meat of these animals was classified as category Z (from animals aged more than 8 months but no more than 12 months), sales description: beef (EC, 2007).

The average daily gain of the animals was high for Holstein calves, ranging from a high of 1.84 kg/d in the L+CLA diet animals down to the slower growth rate of 1.72 kg/d in the control diet animals ($P > 0.05$). In other studies, Holstein calves fed a palm oil supplement attained a growth rate of 1.3 kg/d (Partida, Olleta, Sañudo, Albertí & Campo, 2007), while Italian Holstein calves fed a concentrate admixture that included 5 % whole linseed during the growth period and 8 % whole linseed during the finishing period achieved an average daily gain of 1.21 kg/d (Corazzin, Bovolenta, Sepulcri & Piasentier, 2012). The growth rate of the young bulls in this experiment was also higher than the 1.51 kg/d attained by Holstein bulls fed concentrate admixtures containing 11.2 % whole linseed (Mach et al., 2006). Schlegel et al. (2012) reported no significant differences in the average daily gain of Simmental heifers fed increasing amounts of CLA, and Schiavon, Tagliapietra, Dal Maso, Bailoni & Bittante (2010) likewise found no differences between Piedmontese calves fed a standard feed and others fed that same standard feed enriched with 8 % CLA.

Mean concentrate/admixture intake by the groups was similar (8.3 to 8.9 kg/d), and intake was also similar when expressed in terms of metabolic weight (93 to 98 g DM/$LW^{0.75}$). Concentrate/admixture feed efficiency was also similar, 5 kg/kg LW.

3.2. Carcass quality and composition

No significant differences in carcass weight or carcass classification score (conformation and fat cover) were found by diet (Table 3). Nevertheless, the animals that received L+CLA presented a slightly higher dressing percentage than the animals in the L group. Dressing percentage depends mainly on breed, slaughter weight, sex, and diet and tends to be higher in beef breeds than in dairy breeds (Albertí et al., 2008), rising as slaughter weight increases (Sami, Augustini & Schwarz, 2004). There is little or no difference in dressing percentage between steers and heifers (Bidner et al., 2009; Casas & Cundiff, 2006), with bulls having a higher dressing percentage than heifers but not steers (Albertí, Casasús, Ripoll, Panea & Blanco, 2010), and coarse diets lower the dressing percentage compared to fine diets (Sully & Morgan, 1982). Here the young Holstein bulls had a low dressing percentage, quite similar to other Holstein bulls fed similar diets and slaughtered at similar weights (Mach et al., 2006), but lower than the 54 % for Holstein bulls slaughtered at a higher slaughter weight (577 kg) (Corazzin, Bovolenta, Sepulcri & Piasentier, 2012).

Table 4 summarizes the tissue composition of the 10th rib, considered representative of the tissue composition of the whole carcass (Oliván, Martínez, García, Noval & Osoro, 2001). According to our findings, adding linseed and/or CLA to the diet did not exert any significant effect on 10th rib tissue composition. The high bone fraction values (greater than 20 %) and low muscle fraction values (less than 60 %) were typical for a dairy breed like Holsteins. These young bulls presented a lower percentage

fat value (mean 17.9 %) and higher percentage bone value (mean 22.5 %) than heavier Holstein bulls with a carcass weight of 320 kg fed commercial concentrate without linseed or CLA supplementation, which yielded 19.3 % fat and 21.8 % bone on the 6th rib (Albertí et al., 2008). In meat breeds the percentage muscle is greater than 70 % (Oliván, Martínez, García, Noval & Osoro, 2001) and can even exceed 80 % in double-muscled breeds like Belgian Blue or Piedmontese (Biagini & Lazzaroni, 2005; Schiavon et al., 2011). Greater carcass fatness has been recorded in some studies carried out on steers or cull cows fed concentrate admixtures that included linseed (Kim et al., 2009; Hernandez-Calva et al., 2011), but no such differences were found here. This could be because the diets used here were isoenergetic, while in the papers cited the concentrates supplemented with linseed contained a higher percentage of lipids than the control diets. Work comparing concentrates supplemented with linseed and concentrates supplemented with another lipid ingredient having a similar energy content published by Mach et al. (2006), Kronberg, Scholljegerdes, Lepper & Berg (2011) and Corazzin, Bovolenta, Sepulcri & Piasentier (2012) also found no variation in carcass fatness.

It has been reported in mice that CLA intake can result in decreased fat deposition (Belury, 2002; Park & Pariza, 2007). It has also been reported to lead to a reduction in subcutaneous carcass fat in pigs (Barnes, Winslow, Shelton, Hlusko & Azain, 2012). However, the absence of any effect on fat deposition recorded here is in agreement with the findings of other experiments, in which neither supplementation with 80 g/d of rumen protected CLA for 336 days in bulls (Schiavon et al., 2011) nor supplementation with differing amounts of CLA (2.5, 5, or 10 % of the diet) in sheep (Wynn et al., 2006) gave rise to differences in rib tissue composition.

Table 5 presents the values for the degree of marbling in the *Longissimus dorsi* muscle. The experimental young bulls exhibited low IM fat deposition. Thus,

the percentage marbling area within the *Longissimus dorsi* muscle area was around 3.5, whereas in Holstein steers slaughtered at 26 months the marbling area can reach 25 % and in Wagyu steers the same age 38 % (Albrecht et al., 2011). Comparing the effect of the four diets, no significant differences in marbling were found. Supplementing the diet of calves with linseed, Mach et al. (2006), Kronberg, Scholljegerdes, Lippar & Berg (2011), and Corazzin, Bovolenta, Sepulcri & Piasentier (2012) reported similar results. Similarly, higher marbling of the meat was not recorded for the animals fed the CLA-enriched diet, unlike what has been reported for pigs (Barnes, Winslow, Shelton, Hlusko & Azain, 2012). The sole difference was that there were more marbling flecks in the young bulls supplemented with linseed and linseed + CLA compared to the control group ($P < 0.05$), but this difference did not result in greater marbling in these animals.

3.3. Adipocyte size and lipogenic enzyme activity levels

Adipocyte size and lipogenic enzyme activity levels in the SC and IM fat depots are set out in Table 6. No significant differences in adipocyte size or lipogenic enzyme activity levels owing to the diet were observed for either fat deposit.

Robelin (1981) studied fatty tissue development in Holstein calves at between 15 and 65 % of adult live weight and concluded that subcutaneous fat depot development was attributable mainly to adipocyte hypertrophy. The values for SC fat adipocyte size recorded in this study can be regarded as high and would thus appear to bear out this conclusion. The SC fat adipocyte size values were similar to the values reported for older (18-month-old) Holstein steers by Albrecht et al. (2011), higher than the values for 12-month-old Holstein cattle published by Eguinoa et al. (2003), and higher than the values for the main Spanish breeds at similar ages and growth stages (Alzón, Mendizabal, Arana, Albertí & Purroy, 2007; Mendizabal et al., 1999). Additionally, the ratio of the amount of 10th rib SC fat to 10th rib SC fat adipocyte size carried out in this study yielded a correlation value of 0.30 ($P < 0.05$), attesting to the effect of adipocyte size on SC fat depot development.

Differences in adipocyte size with dietary intake were not observed in the IM fat either. Values were higher than the 20 microns recorded in yearling European x British cross-breed heifers (Mir et al., 2008) but lower than values for Japanese Black steers, a breed highly prone to marbling, where the size of IM adipocytes increased gradually from 60 to 110 microns during the fattening period at between 10 and 26 months of age (Albrecht et al., 2011). In any case, IM fat adipocyte size was much smaller than SC fat adipocyte size (162.3 vs 46.5 µm, $P < 0.001$, mean SC fat and IM fat adipocyte size, respectively, for all four test groups combined). In this connection, IM fat is regarded as a late-developing depot compared with the visceral and SC fat depots (Gotoh et al., 2009; Robelin, 1986).

Fig. 1 and Fig. 2 depict the SC and IM adipocyte size frequency distributions, respectively, for the young bulls according to diet. Fig. 1 shows the SC fat cell distribution to be very similar in the four groups of young bulls and to follow a normal distribution. Fig. 2 also reveals a very similar IM adipocyte distribution in the four dietary groups, but in contrast the distribution was asymmetrical (skewness coefficients: 0.8, 1.2, 0.9, 0.8 for the Control, Linseed, CLA, and Linseed+CLA groups, respectively; $P < 0.05$) with significant numbers of small adipocytes, denoting active adipocyte hyperplasia in the IM depot. This is consistent with the notion that adipocyte hyperplasia predominates in the early stages of fat depot development (the case of the IM fat depot here) and that subsequently adipocyte hypertrophy takes over (the case of the SC fat depot). Therefore, the SC depot was in a more advanced stage of development than the IM fat depot.

Certain researchers have examined the effects of diet on fat depot development. For instance, Yamada & Nakanishi (2012) fed Wagyu steers different dietary roughage/concentrate ratios and observed significant differences in SC fat depot adipocyte size according to the diet administered, with the steers fed a high-roughage/concentrate ratio having larger adipocytes. Barnes Winslow, Shelton,

Hlusko & Azain (2012) fed pigs CLA-enriched diets and also observed an increase in marbling caused by larger IM fat depot adipocyte size. Corino et al. (2007) reported the same effect of CLA supplementation in rabbits. Our diets did not result in any differences in adipocyte hypertrophy with CLA or linseed supplementation, probably because the diets of the different groups was based on concentrate having the same energy and protein content. In this respect, Schoonmaker, Fluharty & Loerch (2004) fed growing Holstein steers diets having different energy contents and found that the steers fed the most energy-rich diets had the largest subcutaneous fat adipocytes. However, when all the groups were fed concentrates having the same energy content during finishing, the differences in subcutaneous fat adipocyte size disappeared.

Turning now to lipogenic enzyme activity, Eguinoa et al. (2003) demonstrated the close relationship between lipogenic enzyme activity and adipocyte size, with adipocyte hypertrophy being accompanied by an increase in enzymatic activity. Accordingly, the lack of differences in lipogenic enzyme activity between the groups of young bulls in this experiment may be attributable to the lack of differences in adipocyte size between the groups (Table 6). Similarly, Schoonmaker, Fluharty & Loerch (2004) showed that energy-rich diets increased lipogenic enzyme activity in steers. Since the diets in this experiment all had the same energy content, it is not surprising that no differences in lipogenic enzyme activity were observed. Finally, Bonnet et al. (2007) reported a higher degree of meat marbling with higher G6PDH lipogenic enzyme activity in steers of different breeds; and since there were no differences in meat marbling in the young bulls in our study, there were similarly no differences in G6PDH enzyme activity. Thus, lipogenic enzyme activity appears to be closely related to adipocyte development, and since the diets tested here had no

effect on adipocyte cellularity they also exhibited no effect on lipogenic enzyme activity.

4. Conclusions

The findings published here indicate that supplementation of an isoenergetic and isoproteic concentrate diet with 10-% linseed and/or 2-% CLA did not result in significant differences in performance, carcass characteristics, or adipose tissue development in young bulls during fattening.

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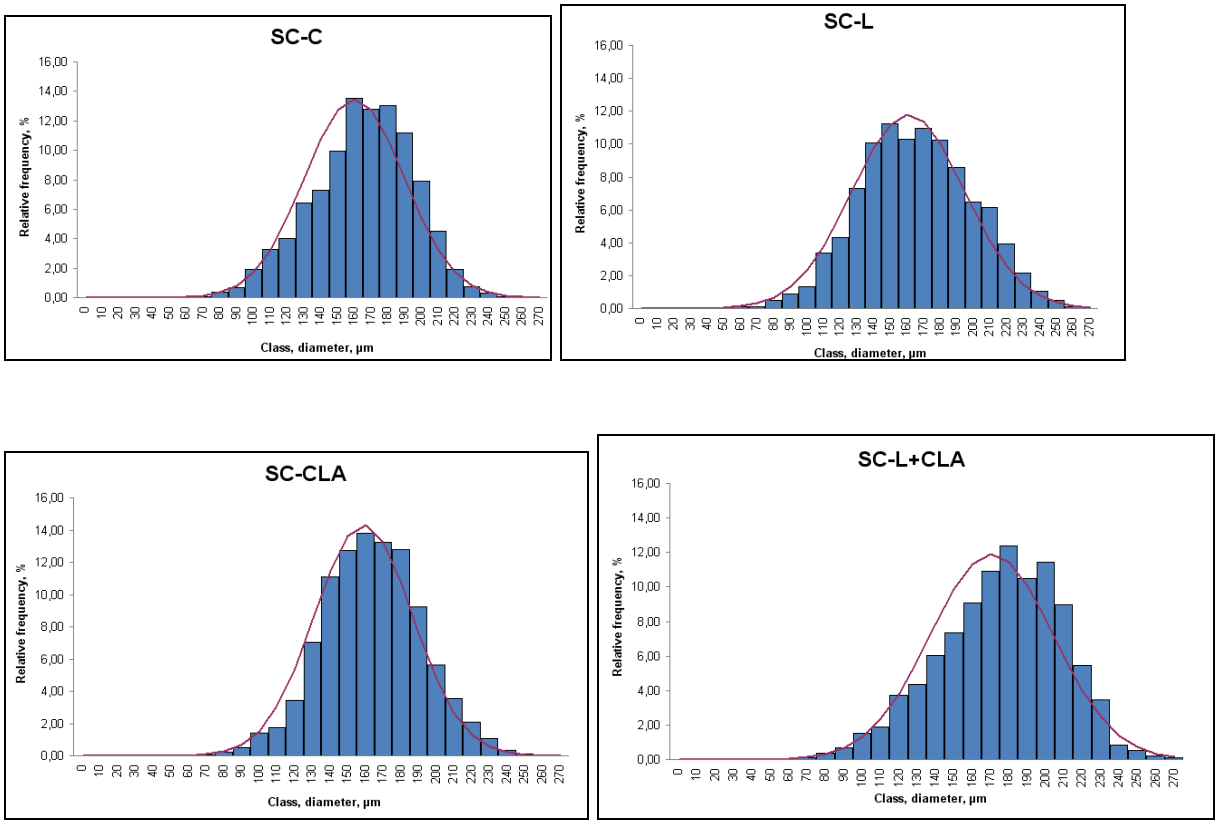

Fig. 1. Subcutaneous adipocyte diameter frequency distributions.

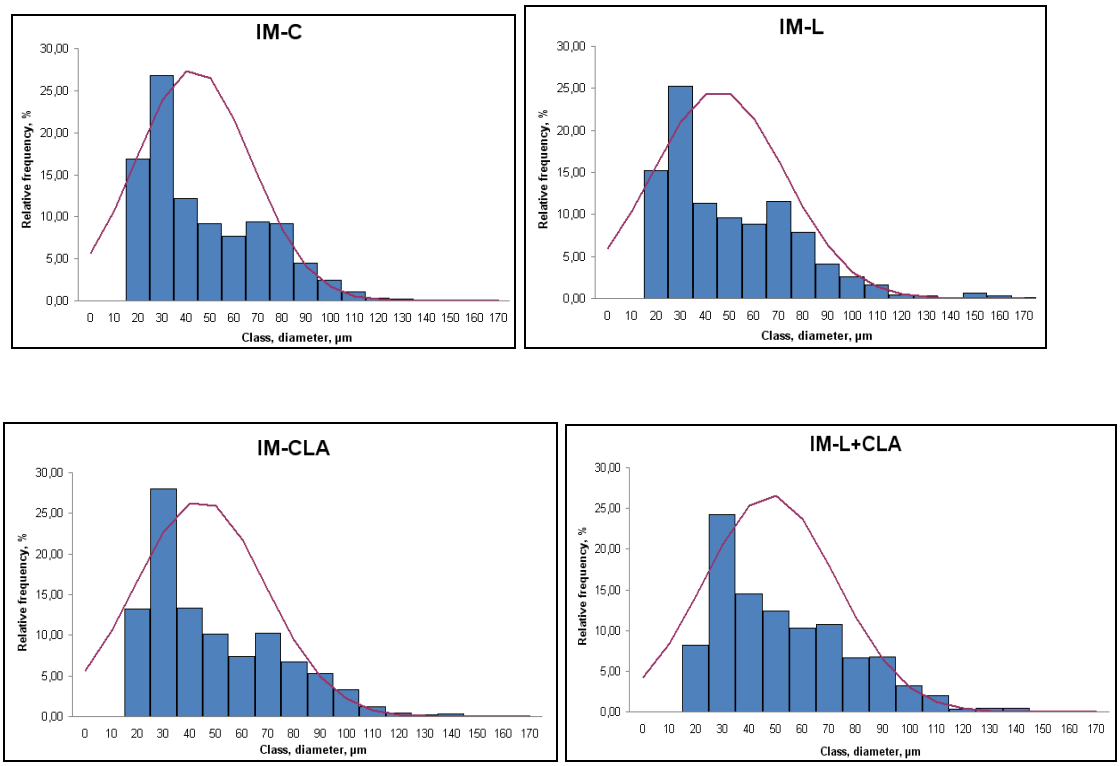

Fig. 2. Intramuscular adipocyte diameter frequency distributions.

Tables

Table 1

Ingredients and chemical composition of the experimental diets

	Diet			
	C	L	CLA	L+CLA
Ingredients (% as fed)				
Maize meal	40.00	40.00	40.00	35.00
Barley meal	21.58	18.77	21.65	19.79
Soybean meal	15.09	13.21	15.07	13.10
Gluten feed meal	12.00	5.00	12.00	6.00
Beet pulp	4.00	9.69	4.00	12.00
Whole linseed		10.00		10.00
Rumen protected CLA			2.00	2.00
Palm oil	4.98	1.16	2.93	
Calcium carbonate	1.23	1.05	1.23	0.99
Salt	0.20	0.20	0.20	0.20
Sodium bicarbonate	0.50	0.50	0.50	0.50
Magnesium oxide	0.20	0.20	0.20	0.20
Mineral-vitamin premix[1]	0.20	0.20	0.20	0.20
Vitamin E[2]	0.02	0.02	0.02	0.02
Proximate analysis				
Crude protein (%)	15.00	15.30	15.00	15.37
Neutral detergent fibre (%)	14.47	15.38	14.48	16.50
Acid detergent fibre (%)	5.57	6.91	5.57	7.44
Ether extract (%)	7.43	6.83	7.35	7.54
Ash (%)	4.85	4.96	4.85	5.08
ME (Mcal/kg)	2.94	2.95	2.94	2.97
Fatty acid profile (% total fatty acid)				
SFA	42.5	21.7	42.0	29.3
MFA	28.1	20.9	26.4	18.3
PFA	29.1	57.2	28.4	49.1

[1] Content per kg: Na_2SO_4 250 g, BHT 150 mg, MgO 50 g, Zn 20 g, Mn 15 g, Fe 2.5 g, Cu 1 g, Co 0.25 g, I 0.25 g, Se 0.1 g, vitamin E 5 mg, vitamin A 3500000 IU, vitamin D3 750000 IU.
[2] Contained 50-% α-tocopherol.
C = Control (concentrate); L = added linseed; CLA = added CLA; L+CLA = added linseed + CLA.

Table 2

Effect of linseed and/or CLA supplementation on performance and intake in young Holstein bulls[1]

	Diet					
	C	L	CLA	L+CLA	SEM	P-value
Initial weight (kg)	239.6	238.9	240.7	240.1	6.38	0.99
Final weight (kg)	450.5	460.4	454.5	468.8	9.77	0.58
Average daily gain (kg/d)	1.72	1.78	1.76	1.84	0.05	0.39
Slaughter age (d)	320.4	326.3	324.1	318.3	5.93	0.77
Concentrate intake[2] (kg/d)	8.3	8.8	8.7	8.9	-	-
Feed efficiency[2] (concentrate kg/weight gain kg)	4.9	5.0	5.0	4.9	-	-

[1]Based on 12 animals/group.

[2]Group parameter.

C = Control (concentrate); L = added linseed; CLA = added CLA; L+CLA = added linseed + CLA.

Table 3

Effect of linseed and/or CLA supplementation on carcass characteristics in young Holstein bulls[1]

	Diet					
	C	L	CLA	L+CLA	SEM	P-value
Cold carcass weight (kg)	233.9	237.8	237.2	249.5	5.67	0.24
Dressing percentage	51.9	51.6[b]	52.2	53.2[a]	0.40	0.04
Conformation score (18 points)	4.2	4.9	4.4	4.8	0.26	0.18
SEUROP classification	O	O	O-	O	-	-
Fat score (15 points)	6.0	5.6	6.2	5.8	0.16	0.06
Fat classification (1-5)	2+	2+	3-	2+	-	-

[1]Based on 12 animals/group.

C = Control (concentrate); L = added linseed; CLA = added CLA; L+CLA = added linseed + CLA.

Different letters in the same row indicate significant differences

Table 4

Effect of linseed and/or CLA supplementation on tissue composition of the 10th rib in young Holstein bulls[1]

	Diet					
	C	L	CLA	L+CLA	SEM	P-value
10th rib weight (g)	1476	1471	1459	1553	48.0	0.55
Tissue composition (%)						
Subcutaneous fat	4.0	3.3	3.6	3.3	0.36	0.45
Intramuscular fat	14.4	14.0	15.2	13.9	0.62	0.46
Muscle	58.4	59.5	57.5	59.7	0.96	0.43
Bone	22.3	22.4	22.9	22.4	0.76	0.92

[1]Based on 12 animals/group.

C = Control (concentrate); L = added linseed; CLA = added CLA; L+CLA = added linseed + CLA.

Table 5

Effect of linseed and/or CLA supplementation on marbling characteristics of the 10th rib[1]

	Diet					
	C	L	CLA	L+CLA	SEM	P-value
Longissimus dorsi muscle area (cm^2)	64.0	68.0	72.2	71.1	2.31	0.09
Intramuscular fat area (cm^2)	2.17	2.48	2.52	2.66	0.21	0.47
IM fat/LM area (%)	3.37	3.73	3.52	3.73	0.31	0.84
Size of marbling flecks (mm^2)	12.3	11.6	13.3	11.1	1.07	0.54
Number of marbling flecks	18[b]	23[a]	19	24[a]	1.64	0.04

[1]Based on 12 animals/group.

C = Control (concentrate); L = added linseed; CLA = added CLA; L+CLA = added linseed + CLA.

Different letters in the same row indicate significant differences

Table 6

Effect of linseed and/or CLA supplementation on adipocyte diameter and lipogenic enzyme activity[1]

	Diet					
	C	L	CLA	L+CLA	SEM	P-value
Adipocyte diameter (µm)						
SC	160.0	160.0	159.7	169.4	6.85	0.70
IM	45.6	48.1	43.6	48.8	2.94	0.58
G3PDH activity (nmol/min/10^6adip)						
SC	290.3	207.6	255.4	321.2	38.29	0.20
IM	170.2	206.1	125.8	252.7	41.17	0.24
FAS activity (nmol/min/10^6adip)						
SC	173.0	167.4	185.9	193.9	25.14	0.88
IM	105.5	124.3	69.5	126.9	12.45	0.25
ICDH activity (nmol/min/10^6adip)						
SC	3743.4	3579.8	3316.3	3622.1	536.38	0.96
IM	3044.0	2330.4	2434.4	2406.2	516.02	0.85
G6PDH activity (nmol/min/10^6adip)						
SC	422.5	485.3	513.9	528.7	85.19	0.81
IM	231.4	230.1	154.1	205.9	62.82	0.86

[1]Based on 12 animals/group.

C = Control (concentrate); L = added linseed; CLA = added CLA; L+CLA = added linseed + CLA.

4. Ácidos grasos

Fatty acid composition of young Holstein bulls fed whole linseed and rumen-protected conjugated linoleic acid enriched diets[1]

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ABSTRACT: Forty-eight young Holstein bulls were used to evaluate the effect of whole linseed and conjugated linoleic acid (CLA) supplementation on beef fatty acid profile in intramuscular and subcutaneous fat. Animals were fed one of four isoenergetic and isoproteic diets: control (0% linseed, 0% CLA), linseed (10% linseed, 0% CLA), CLA (0% linseed, 2% CLA), and linseed plus CLA (10% linseed, 2% CLA). The fatty acid profile had similar trends in intramuscular and subcutaneous fat when diets were enriched with linseed and/or CLA, increasing the level of 9c11tCLA and α-linolenic acid, and decreasing the *n-6*/*n-3* fatty acid ratio. Supplementation with linseed improved the fatty acid profile by increasing the proportions of *n-3* and CLA fatty acids and decreasing the *n-6*/*n-3* ratio compared with the control. CLA addition achieved similar CLA tissue levels to linseed supplementation, but similar *n-3* proportions to the control. Linseed plus CLA supplementation was more effective in increasing total polyunsaturated fatty acids and CLA compared with their individual addition. It is concluded that supplementation of whole linseed and/or CLA in bulls resulted in an increase in beef fat of some fatty acids considered to be of benefit of human health.

Key words: *n-3*, CLA, α-linolenic acid, beef, intramuscular fat, subcutaneous fat

INTRODUCTION

The manipulation of the fatty acid (FA) composition in ruminant fat to improve its profile is of major importance in meat research. Some of the *n-3* polyunsaturated fatty acid (PUFA) and conjugated linoleic acid (CLA) provide advantages to human health (Mir et al., 2003). The *n-6*/*n-3* fatty acid ratio depends on the content of fractions of phospholipids and triglycerides, and it is more affected by the diet than the animal genetics (Mir et al., 2003). Thus, the supplementation of ruminant diets with PUFA rich lipids is the most effective approach to decrease saturated fatty acids (SFA) and promote the enrichment of meat with CLA and *n-3* PUFA.

Linseed is a natural source of linolenic FA, and its seed coat may provide some protection to PUFA against rumen biohydrogenation and thus increase the passage of PUFA into the duodenum. The feeding with flaxseed on beef has been studied by several authors (Maddock et al., 2006; Razminowicz et al., 2008; Scholljegerdes and Kronberg, 2010; Kronberg et al., 2011; He et al., 2012; Kronberg et al., 2012; Corazzin et al., 2013; Mapiye, Aalhus, et al., 2013; Mapiye, Turner, et al., 2013). Moreover, in cattle, to prevent rumen hydrogenation of the CLA, it must be supplied in rumen-protected forms (Perfield et al., 2004). There are some studies that have evaluated the effect of the inclusion of rumen-protected CLA on the FA profile of meat (Gillis et al., 2004; Poulson et al., 2004; Gillis et al., 2007; Schiavon et al., 2011; Schlegel et al., 2012).

Nevertheless, there is limited information available on the effect of the addition of whole linseed plus rumen-protected CLA on the FA profile and the increased quantities of FA of nutritional interest on beef. Thus, the objective of the

current study was to examine the effects of feeding a concentrate diet including whole linseed, rich in *n-3* fatty acids, and/or protected CLA on the fatty acid composition of intramuscular fat and subcutaneous adipose tissue of beef from young Holstein bulls.

MATERIALS AND METHODS

The study was performed at the CITA experimental farm in Aragón (Spain), where animal care and use practices were in accordance with EU Directive 2010/63/EU of 22 September 2010 on the protection of animals used for scientific purposes.

Animals and feeding

Forty-eight Holstein entire males (initial live weight 239.8±0.7 kg and 198.7±4.1 days old) were allocated randomly to 8 different groups housed in different pens of six animals each (2 pens per dietary treatment). The four concentrate diets contained the same feed ingredients and supplementation with vitamin E (110 mg/ kg concentrate) (Table 1). The animal diets were formulated to be isoenergetic and isoproteic and they differed in the percentage of whole linseed and protected CLA (Lutrell® pure, BASF, Germany). All of the groups were fed their corresponding concentrate as described below: two control groups (C, 6 animals per group; 0% linseed, 0% CLA), two groups fed whole linseed (L, 6 animals per group; 10% linseed, 0% CLA), two groups fed CLA (CLA, 6 animals per group; 0% linseed, 2% CLA) and two groups fed whole linseed plus CLA (L+CLA, 6 animals per group; 10% linseed, 2% CLA). Feed samples were collected monthly and lipids were extracted and methylated as described by Albertí et al. (2013). After a finishing period of 123±11.2 days, the bulls (mean live weight 458.4±16.6 kg) were

slaughtered at an EU-licensed commercial abattoir following standard procedures. Animal productive performance and carcass characteristics of these animals were reported by Albertí et al. (2013).

Slaughter and sample collection

Slaughter weight was set at 450 kg live weight. The animals were transported 10 km for slaughter at an EU-licensed commercial abattoir where they were dressed according to commercial practice.

At 24 h after slaughter, *Longissimus thoracis* (LT) steaks (100 g) and a sample of subcutaneous adipose tissue (10 g) were cut at the 6th rib level from the left half-carcass for the intramuscular and subcutaneous fatty acid composition analysis. Fat and meat samples were vacuum-packaged in pouches of polyamide/polyethylene (120 mm and 1 cc/m^2 per 24 h O_2 permeability, 3 cc/m^2 per 24 h CO_2 permeability and 0.5 cc/m^2 per 24 h N_2 permeability, measured at 5ºC and 75% relative humidity) (Vaeseen Schoemarket Ind. Spain) and frozen and stored at -20ºC until lipid composition analysis. Before analyses, the steaks and SC adipose tissue samples were thawed at 4º C overnight.

Fatty acid analysis

Total lipids from beef were extracted and hydrolysed as described by Whittington et al. (1986) with some modifications by Aldai et al. (2005). All of the samples were analyzed in duplicate. The extracted fatty acids (FAs) were methylated using 200 μL of trimethylsilyl-diazomethane at 40 °C for 10 min, dried under N_2, dissolved, centrifuged and the supernatant transferred for analysis. The FA methyl esters were stored at −80 °C for later chromatographic analysis by gas chromatography using a BPX-70 (SGE U.K. Ltd) fused-silica capillary column (120

m×0.22 mm i.d.× 0.2 μm film thickness). The FA methyl esters were separated by gas chromatography 7890A using a flame ionization detector (FID) and hydrogen as the carrier gas. The oven temperature was initially set at 50 ºC, and gradually ramped up 240 ºC where it remained to the end of the cycle. The entire process took about 50.5 min. FA methyl esters were identified by retention times, using standards where available (Sigma Chemical Co. Ltd., Poole, UK; Nucheck, Elysian, USA). Fatty acid profiles were expressed as a percentage of the total fatty acids identified.

Fatty acids were quantified using tricosanoic acid methyl ester (C23:0), added prior to saponification, as an internal standard. Column response and linearity were checked using a mixture of fatty acids (C16:0, C18:0, C18:1n9, C18:2n6, relative to internal standard C23:0, Sigma Chemical Co. Ltd., Poole, UK; Nucheck, Elysian, USA). Fatty acids quantities were expressed as mg FA / 100 g muscle.

Statistical analysis

Data were analyzed as a completely randomized design using a general linear model (GLM) procedure (IBM-SPSS version 19 for Windows, 2011). The effect of pen (replicate) was not significant between the two groups from each the dietary treatments. Therefore, the statistical model included the fixed effects of diet and tissue, as well as diet × tissue interaction and residual error. When diet × tissue significant interactions were detected, differences between means were further analyzed by the Tukey's test. Differences between means were considered to be significant at $P<0.05$. In order to assess the differences among diets and to determine the contribution of fatty acids to these differences, a canonical discriminant analysis based on individual fatty acids percentages was performed for both depots

(intramuscular and subcutaneous). Discriminant analysis was performed using a discriminant procedure (IBM-SPSS version 19 for Windows, 2011).

RESULTS AND DISCUSSION

Fatty acid profile of the experimental diets

The profile of the main fatty acids of the different diets is shown in Table 2. Including linseed in the diet (diet L and diet L+CLA) markedly increased the C18:3n3 (ALA) content at the expense of C16:0. Therefore, in the diets L and L+CLA the PUFA total content was significantly higher, whereas their SFA total content was significantly lower, compared with the diets C and CLA. The C18:2n6 (LA) percentage was similar in the C, CLA and L+CLA concentrates (25–27 g/100 g of total fatty acids), whereas in the L concentrate was significantly higher (35 g/100 g of total fatty acids). Moreover, including CLA in the diet markedly increased the RA, 10t12cCLA and 9t11tCLA content compared with the C and L concentrates. The addition of linseed plus CLA (diet L+CLA) showed the lowest 18:1c9 content, and consequently total monounsaturated fatty acid (MUFA) content, compared with the others diets (C, L and CLA).

Tissue fatty acid profile

The percentage of intramuscular fat (IM fat area/muscle area) from the young Holstein of the current study ranged around 3.6% (Albertí et al., 2013).

Polyunsaturated fatty acids. Table 3 shows the PUFA proportions (%) by adipose tissue, animal diet and their interaction. There was no interaction D x T ($P>0.05$) for total PUFA. The effect of diet was significant ($P<0.05$) for the total PUFA. In the IM tissue, young bulls fed diets containing L plus CLA had higher

proportions of total PUFA than bulls fed diets containing whole linseed, CLA or control diet. In the SC tissue, young bulls fed L and L+CLA diets had higher proportions of total PUFA than bulls fed CLA or Control diets.

Differences in the total PUFA between the IM and the SC tissue were significant (P<0.001), with higher concentrations of total PUFA in the IM (15.53%) than in the SC (3.75%) tissue. This higher proportion of PUFA in the IM tissue could be partly attributable to the fact that the membrane/cytoplasm relation is lower in IM tissue because the adipocytes size was lower in the IM tissue (Albertí et al., 2013), as well as membrane phospholipids in the IM tissue show higher proportion of PUFA (Raes et al., 2004).

The fatty acid LA (C18:2n6c9c12) was the predominant *n-6* contributing around 71% and 68% of total *n-6* in IM and SC tissues, respectively. Diet x Tissue interaction was not significant (*P*>0.05) for LA. Besides, there were no significant differences among diets (*P*>0.05) having similar proportions of LA in the IM (9.62%) and the SC (1.91%) tissues.

The proportions of LA were only influenced by the tissue (*P*<0.001) and were 5-fold higher in the IM than the SC tissue. In the IM depot the proportions of LA (8.37-10.24%) were similar to those obtained in Holstein bulls fed concentrates containing linseed (Mach et al., 2006), and higher than those obtained in beef heifers offered pasture supplemented with linseed oil rich concentrates (Noci et al., 2007) and in steers fed red clover silage with and without flaxseed (Mapiye, Aalhus, et al., 2013). The proportions of LA in the SC tissue of the bulls averaged 1.91% and were higher than those reported by Noci et al. (2007) and Mapiye, Aalhus, et al. (2013).

ALA (C18:3n3) was the most predominant fatty acid of the *n-3* family contributing around 57% and 100% of total *n-3* in IM and SC tissues, respectively. The effects of diet and tissue and their interaction influenced the proportion of ALA, (P<0.001). Bulls fed the diet containing 2% CLA had similar proportion of ALA compared to those fed the Control diet in the IM and the SC tissues. However, L and L+CLA diets increased around 8-9–fold the proportion of ALA in both tissues. These results are due to the large difference in ALA content among diets (Table 2). The proportions of ALA in the IM tissue of bulls fed the L and L+CLA diets in the current study were higher than those reported by other authors using flaxseed containing diets (Aharoni et al., 2004; Mach et al., 2006; Noci et al., 2007; Juárez et al., 2011; Mapiye, Turner, et al., 2013). The levels of ALA obtained in the SC tissue of bulls fed containing whole linseed (L, 0.79%; L+CLA, 0.90%) were slightly lower to those reported by (Mapiye, Turner, et al., 2013), but greater than those reported when beef cattle were fed either forage-based diets supplemented with flaxseed (Aharoni et al., 2004; Noci et al., 2007; Nassu et al., 2011; He et al., 2012).

The proportions of ALA in the IM tissue of bull fed diets L and L+CLA were similar, whereas the addition of whole linseed plus CLA resulted in higher numerical proportions of LA, and higher PUFA (P<0.05) compared with the L diet. This could be partly attributed to the different FA proportions among the four diets (Table 2). The L and L+CLA diets had similar proportions of PUFA (53.17%) and ALA (22.86%), but different proportions of LA (34.84% *vs* 25.45%). These results could be related to that ALA has a higher level of biohydrogenation in the rumen than LA (Doreau and Ferlay, 1994), whereas LA seems to have an inhibitory effect on its own biohydrogenation (Mapiye, Aalhus, et al., 2013). It may also be a result of

competition between ALA and LA for common metabolic enzymes (i.e., desaturases and elongases) and for incorporation into cell membranes (Raes et al., 2004). Chow et al. (2004) demonstrated that in vitro biohydrogenation of LA from sunflower oil or linseed oil averaged 75%, whereas biohydrogenation of ALA from linseed oil averaged 84% under the same conditions.

In the IM tissue, EPA (C20:5n3) and DPA (C22:5n3) were influenced by diet ($P<0.001$, Table 3), whereas DHA (C22:6n3) did not differ ($P>0.05$) among dietary treatments. Bulls fed the diet containing 2% CLA had similar proportions of EPA, DPA and DHA to those fed the Control diet ($P>0.05$). These results are comparable to those reported by Mapiye, Turner, et al. (2013) and Nassu et al. (2011) in bulls fed diets that were not enriched with long-chain fatty acids. In contrast, whole linseed supplementation in L and L+CLA diets increased twice the proportions of EPA (0.27%) and DPA (0.46%), although DHA was not influenced. In agreement with current results, previous studies (Noci et al., 2007; Nassu et al., 2011; Mapiye, Aalhus, et al., 2013) reported these increases in EPA and DPA in beef tissues with diets rich in ALA. In contrast, Mapiye, Turner, et al. (2013) did not report these increases in steers fed flaxseed in a red clover silage diet. Similar to the present findings, other authors found no increases in DHA when feeding flaxseed to beef cattle (Noci et al., 2007; Juárez et al., 2011; Nassu et al., 2011; Mapiye, Aalhus, et al., 2013; Mapiye, Turner, et al., 2013). This may be a consequence of the competition between ALA and the precursor for DHA (i.e., 24:5n3) for the activity of the Δ6 desaturase enzyme (Cameron et al., 2000). The proportions of EPA, DPA and DHA in young bulls fed L and L+CLA diets of the current study were higher than those reported by Mapiye, Turner, et al. (2013) in intramuscular fat of beef

steers fed red clover silage with flaxseed and lower than those reported by Noci et al. (2007).

Diet x Tissue interactions affected the proportions of total CLA and the main CLA isomers (9c11t CLA and 10t12cCLA) (P<0.05). Diet influenced the CLA isomers content (P<0.001), except 9c11cCLA (P=0.098). The type of adipose tissue also influenced the proportions of the identified CLA isomers (P<0.05), except for the 9c11cCLA (P=0.179), and these proportions were higher in SC tissue compared with the IM tissue.

RA was the predominant CLA isomer and contributed 43–62% and 56–67% of total CLA isomers in IM and SC tissues, respectively. The 10% whole linseed supplementation (L diet) increased twice the proportions of RA in both tissues compared with the Control diet. This could be related to ALA and the LA have been increased in the diet and by the process of biohydrogenation of both, that it is conducted in the rumen by the *Butyrivibrio fibrosolvens* bacterium, RA is generated and subsequently deposited in the tissues (Kepler et al., 1966). This high content of RA may also be due to the intervention of the reductase enzymes, from the bacteria in the rumen, that are capable of reducing the RA to VA (t11 vaccenic, C18:1t11), and once in the tissues, by the action of the enzyme Δ9-desaturase (Griinari and Bauman 1999) is converted to RA. In previous studies the high content of CLA in beef has been related with the high content of LA (Madron et al., 2002; Mir et al., 2002; Mir et al., 2003; Noci et al., 2005; Noci et al., 2007) and ALA from the diet (Enser et al., 1999; Stasiniewicz et al., 2000; Raes et al., 2003). Similarly, the 2% CLA supplementation increased twice the proportion of RA in IM and SC tissues from bulls fed the CLA diet compared to those tissues from bulls fed the C diet.

These results indicated that the direct supply of rumen-protected CLA in the diet was as effective as the supply of enough dietary precursors to increase the proportions of CLA in tissues. Furthermore, in the IM and SC tissues from young bulls fed L+CLA diet, the proportion of RA was increased 3-fold compared to those tissues from bulls fed Control diet.

Summarizing, animal diet influenced the proportions of PUFA in beef from Holstein bulls in the current study. Addition of 10% whole linseed improved the fatty acid profile of beef compared with the CLA and Control diets. Addition of 10% whole linseed plus 2% CLA showed a additive effect achieving higher proportions of total PUFA and CLA compared with the other dietary treatments. It should be noted that differences in live weights and age at slaughter, breeds, concentrates, sources of linseed and CLA, make it difficult to compare these results to those obtained by other authors.

Monounsaturated fatty acids. Table 4 shows the MUFA proportions (%) by adipose tissue, animal diet and their interaction. Diet x Tissue interaction was not significant for total MUFA ($P>0.05$). Monounsaturated fatty acids were influenced by the tissue ($P<0.001$) and were higher in the SC tissue compared to the IM tissue (45.26 *vs* 36.68%, total MUFA).

Bulls fed L diet tended ($P=0.077$) to have higher proportion of total MUFA than bulls fed CLA, L+CLA or C diets. Oleic acid (C18:1c9) was the predominant MUFA in IM and SC tissues contributing around 75% of total MUFA.

MUFA including oleic acid are considered to be beneficial for human health by reducing inflammation and blood coagulation factors (Williamson et al., 2005), but the effects of the minor MUFA on human health are not known and warrant

further research. Furthermore, VA could have additive health effects directly (Jacome-Sosa et al., 2010) or by desaturation to RA, that has potential to reduce cardiovascular diseases, cancer and diabetes (Benjamin and Spener, 2009). Bulls fed L+CLA diet had the highest proportion of VA in both tissues in the current study. Previous studies also reported this effect of accumulation of VA in cattle fed a combination of oils or oilseeds rich in ALA and LA (AbuGhazaleh et al., 2002). This could be related with the LA, which is the main precursor of CLA and a competitive inhibitor of the biohydrogenation of VA (Agazzi et al., 2004). Harfoot et al. (1973) reported that high levels of LA inhibit biohydrogenation of VA produced and Moate et al. (2008) found that if the concentration of VA exceeds 517 ppm in rumen fluid, VA auto–inhibits its own biohydrogenation to stearic. This would increase the amount of VA into tissue. Notably, the proportion of VA in SC fat when young bulls were fed L+CLA diet was two to three times higher than previously reported values when feeding flaxseed in high-forage diets (Nassu et al., 2011; He et al., 2012), and it was similar when feeding flaxseed in a diet with a high level of red clover silage (Mapiye, Turner, et al., 2013).

Saturated fatty acids. Table 5 shows the SFA proportions (%) by adipose tissue, animal diet and their interaction. Diet x Tissue interaction was not significant (P=0.852) for total SFA. Diet effect was significant (P=0.001) and the proportions of total SFA were higher in bulls fed Control diet compared to those fed CLA, L+CLA and L. The differences are mainly due to the palmitic (C16:0) that was the predominant SFA contributing around 50% of total SFA. The diet effect was not significant (P>0.05) for stearic (C18:0, the second predominant SFA) or myristic (C14:0). The proportions of stearic were higher in IM tissue than SC tissue (17.82 vs

15.51%; P<0.001), whereas the proportions of palmitic were higher in SC tan IM tissue (28.08 vs 24.94%; P<0.001), resulting in a higher degree of saturation in the SC than the IM fat depot.

Supplementation with 10% whole linseed and/or 2% CLA in the present experiment resulted in a decrease in the SFA in the IM and SC adipose tissue. An increase in the dietary supply of sunflower oil or linseed oil had a similar effect of decreasing SFA in beef cattle (Noci et al., 2007). The decrease in SFA seems to be mainly due to a decrease in the proportion of palmitic acid (Noci et al., 2007) as result of the increase in the deposition of PUFA in beef. This is agreement with previous studies (Nassu et al., 2011; Mapiye, Turner, et al., 2013) that reported decreases in the proportions of C16:0 in the muscle and adipose tissue in steers fed flaxseed plus forages. These authors attributed the decrease in C16:0 to the supresion of lipogenic genes (for instance, fatty acid synthase and stearoyl-CoA desaturase) responsible for the endogenous synthesis of FA from ALA provided by flaxseed. The lower proportions of C16:0 obtained in young bulls fed whole linseed and/or CLA (L, CLA and L+CLA diets) compared to control (C diet) may also be explained by dietary differences in C16:0 between the four diets (Table 2). Current findings indicate that whole linseed and/or CLA addition in animal concentrate diets can improve the fatty acid profile of beef from young Holstein bulls.

Fatty acid ratios. Table 6 shows FA ratios of nutritional interest by adipose tissue, animal diet and their interaction. Intramuscular fat from bulls fed L+CLA diet had 0.38 for the PUFA/SFA ratio and it was higher than in bulls fed L, CLA or C diets. On the other hand, the SC tissue from bulls fed L and L+CLA diet increased

twice the PUFA/SFA ratio, because the proportions of SFA decreased and the proportions of PUFA increased.

Although 10% whole linseed plus 2% CLA supplementation improved the PUFA/SFA ratio in muscle from young bulls of the current study, this ratio was lower than recommendations (>0.45; Department of Health, UK, 1994). In the present experiment, L diet did not improve the PUFA/SFA ratio in the IM tissue, although other previous studies improved this ratio by linseed addition. For instance, Mach et al. (2006) increased PUFA/SFA ratio in fat from bulls fed diets containing 11.2% or 18% (0.38 and 0.39, respectively) linseed compared to those fed diets containing similar proportions of canola seed (0.29). However, comparisons of the PUFA/SFA ratio across studies should be made with caution because lean animals will have a greater PUFA/SFA ratio irrespective of ration composition (Raes et al., 2003). CLA diet did not improve the PUFA/SFA ratio in the two tissues studied, and these findings are in agreement with the results obtained in bulls fed diets containing 2% rumen-protected CLA salt (Gillis et al., 2004). Moreover, SC tissue from bulls fed L and L+CLA diet increased twice the PUFA/SFA ratio, because the proportions of SFA decreased and the proportions of PUFA increased, so the PUFA/SFA ratio (0.095) was improved.

The *n-6*/*n-3* ratio of beef is of relevance in its contribution to the whole diet of humans. The effect of diet and the *D* x *T* interaction influenced the *n-6*/*n-3* ratio ($P<0.001$), whereas there was no tissue effect ($P>0.05$).

The *n-6*/*n-3* ratio of beef from bulls fed the Control diet was the highest among all dietary treatments. The 2% CLA addition resulted in the decrease of n-6/n-3 ratio (around 20), although it remained higher than nutritional recommendations (4;

Simopoulos, 2008). The 10% whole linseed supplementation increased *n-3* and therefore, decreased significantly the *n-6*/*n-3* ratio in both tissues (around 4). The *n-6*/*n-3* ratios of beef from Holstein fed L and L+CLA diets were lower than those obtained in Friesian cattle fattened with diets containing 3.6%, 11.2% and 18.0% of flaxseed (Mach et al., 2006). The results obtained in this study show that the main differences found between *n-6*/*n-3* ratios depended on the addition of whole linseed in L and L + CLA diets, which resulted in lower *n-6*/*n-3* ratios due to higher proportions of *n-3*. Furthermore, it has been found that the increases in *n-3* fatty acids in meat, by linseed oil or fish oil supplementation in diets, decreases *n-6*/*n-3* ratio but has little influence on the PUFA/SFA ratio (Scollan et al., 2001). Scollan et al. (2003) proposed a negative exponential relationship between the amount of intramuscular fat and the PUFA/SFA ratio.

Discriminant analysis. The canonical discriminant analysis (CDA) produced three discriminant functions. Function 1 accounted for 79.2% of the total variability among diets and was mainly determined by C18:2n6t9t12, ALA, iC17:0, CLA9c10c, C20:3n6, C18:3n6 and C15:0 proportions. Function 2 accounted for 16.4% of total variability and was characterized by CLA9t11t, CLA9c11t, C18:1t11, C16:0, C16:1c9, C18:0, C14:1c9, C17:0, C15:1, C20:0 and C14:0.

The results indicate that the 97.90% of grouped cases were correctly classified into the corresponding dietary treatments. Most of the animals were in the diagonal, so this indicates that there was agreement between the assignment into groups by CDA method and the real grouping of the animals.

Figure 1 shows that animals were separated in four groups according to the supplied diet (C, L, CLA and L+CLA) in terms of their fatty acid profile, illustrating

that there is an effect of the diet on the FA profile in IM and SC tissues from young Holstein bulls in the current study. Moreover, these results evidence that the CDA can be used to discriminate the relationships among FA composition of beef from bulls and their diets. In previous studies, CDA has been used successfully to discriminate among four types of diet fed cattle during the finishing period (Alfaia et al., 2009) or to distinguish beef samples according to the type of finishing diet fed to bulls (Martínez Marín et al., 2013).

Fatty acids of nutritional interest

Table 7 shows the quantities (mg FA / 100 g muscle) of the FA of nutritional interest for their positive effects on human health. The *n-3* fatty acids ALA, EPA and DHA provide a wide range of benefits from general improvements in health to protection against inflammation and disease (Ganesan et al., 2014), and CLA has been identified as possessing anticancer properties and other positive health properties (Dilzer and Park, 2012).

The total fatty acid content of the muscle averaged 1848.98 mg/100 g of muscle and there were no significant differences among diets (P=0.260).

The fatty acid LA was the predominant *n-6* contributing around 63% of total *n-6* in IM tissue. There were no significant differences among diets (P=0.422) having similar content (104.90 mg LA/100 g muscle). A ration of 200 g of beef from bulls of the present study would supply 209.80 mg of LA, compared with a recommended intake of 17 and 12 g/d for men and women, respectively (Institute of Medicine of the National Academies, 2002).

ALA was one of the predominant fatty acids of the *n-3* family contributing from 16% to 40% of total *n-3* in the IM tissue. The contents of ALA in bulls fed L

and L+CLA diets were 6-fold higher than those fed Control and CLA diets (12.89 *vs* 1.96 mg /100 g muscle; $P<0.001$). A ration of 200 g of beef from bulls fed C and CLA diets would provide approximately 3.92 mg of ALA, whereas beef from bulls fed linseed plus CLA enriched diets would increase this quantity up to 25.78 mg. However, although a considerable increase in the ALA was achieved in the current study, the absolute contribution of such beef to the recommended consumption of ALA is small (1.6 g/d for men and 1.1 g/d for women; Institute of Medicine of the National Academies, 2002).

The contents of EPA plus DHA in bulls fed L and L+CLA diets were twice higher than those fed Control and CLA diets (6.29 *vs* 3.44 mg /100 g muscle; $P<0.001$). However, the levels of EPA plus DHA were increased by the whole linseed supplementation but the quantities of these FAs were lower from the intake of 250 mg *n-3* long chain fatty acids per day suggested as the minimum needed for the promotion of cardiovascular health (Musa-Veloso et al., 2011). Thus, 200 g of beef from young bulls fed Control and CLA diets would contributed the 2.8% of the adequate intake of EPA plus DHA, whereas beef from young bulls fed L and L+CLA diets increased this intake up 5.0%.

The average intake of CLA from natural sources, primarily the RA, is estimated as 151–212 mg/d for Americans and 97.5 mg/d for the British (Ritzenthaler et al., 2001; Mushtaq et al., 2010; Dilzer and Park, 2012). Based on these data, a ration of 200 g of beef from young bulls fed Control diet would provide 4.58 mg of RA; this means that it would contribute 2.52 to 4.70% of the average intake of RA for consumers. Whole linseed or CLA supplementation in L and CLA diets increased the contribution 4.26 to 7.93% of the dietary CLA intake mentioned

previously. The quantities of CLA in bulls fed L+CLA diet was the highest contribution (6.53 to 12.16%). But actually, this contribution is greater given that about 30% of VA is converted to RA in humans, it has been recommended that true dietary intake of CLA should consider native RA as well as the VA (potential RA) content of foods (Daley et al., 2010). So, including VA conversion, *longissimus dorsi* from young bulls fed L+CLA diet from the present study could therefore contribute 15.23 to 28.35% (VA, 8.70 to 16.19% + RA, 6.53 to 12.16%) of the average intake of RA for consumers. Thus, the enrichment of diets with whole linseed plus CLA, would result in beef with higher RA contents than those of conventional beef, and which would be closer to intake considered necessary for cancer prevention (0.095 g/d, Knekt et al., 1996; 3 g/d, Ip et al., 1994). It should be noted that nowadays further research is being conducted to unveil its benefits, recommended doses and target population groups (Dilzer and Park, 2012).

To sum up, in view of the beneficial effects of *n-3* PUFA and CLA, the increases of these FAs in the muscle, due to whole linseed and CLA supplementation in the bulls of the current study, can be considered a positive finding.

Conclusion

The intramuscular fat showed higher proportions of total polyunsaturated and *n-3* fatty acids and lower proportions of CLA, total monounsaturated and saturated fatty acids than the subcutaneous fat in beef from bulls fed all dietary treatments. However, the fatty acid profile had similar trends in both tissues when diets were enriched with whole linseed and/or CLA, increasing the level of 9c11tCLA and α-linolenic acid, and decreasing the *n-6*/*n-3* fatty acid ratio. Supplementation with 10% linseed improved the fatty acid profile by increasing the proportions of *n-3* and CLA

fatty acids and decreasing the *n-6*/*n-3* ratio compared with the control commercial diet. Addition of 2% CLA achieved similar CLA tissue levels to addition of 10% linseed, but similar *n-3* proportions to the commercial diet. Linseed (10%) plus CLA (2%) showed an additive effect increasing the proportions of total polyunsaturated fatty acids and CLA compared with their individual addition. Dietary supplementation with 10% linseed is recommended to increase the *n-3* and CLA fatty acids in beef from young Friesian bulls with further improvements in CLA by adding 2% CLA, resulting in *n-6*/*n-3* ratios and intakes of the fatty acids of nutritional interest in beef closer to nutritional recommendations for a healthy diet. Although the *n-3* and CLA enriched beef would have to greater price to offset the greater feed costs associated in the diet, this study demonstrates that it is possible to develop beef enriched with *n-3* and CLA from bulls through whole linseed plus CLA.

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Table 1. Ingredients and chemical composition of the experimental diets.

	C	L	CLA	L+CLA
Ingredients (% as fed)				
Maize meal	40.00	40.00	40.00	35.00
Barley meal	21.58	18.77	21.65	19.79
Soybean meal	15.09	13.21	15.07	13.10
Gluten feed meal	12.00	5.00	12.00	6.00
Beet pulp	4.00	9.69	4.00	12.00
Whole linseed		10.00		10.00
Rumen protected CLA			2.00	2.00
Palm oil	4.98	1.16	2.93	
Calcium carbonate	1.23	1.05	1.23	0.99
Salt	0.20	0.20	0.20	0.20
Sodium bicarbonate	0.50	0.50	0.50	0.50
Magnesium oxide	0.20	0.20	0.20	0.20
Mineral-vitamin premix[1]	0.20	0.20	0.20	0.20
Vitamin E[2]	0.02	0.02	0.02	0.02
Proximate analysis				
Crude protein (%)	15.00	15.30	15.00	15.37
Neutral detergent fibre (%)	14.47	15.38	14.48	16.50
Acid detergent fibre (%)	5.57	6.91	5.57	7.44
Ether extract (%)	7.43	6.83	7.35	7.54
Ash (%)	4.85	4.96	4.85	5.08
ME (Mcal/kg)	2.94	2.95	2.94	2.97

[1] Content per kg: Na_2SO_4 250 g, BHT 150 mg, MgO 50 g, Zn 20 g, Mn 15 g, Fe 2.5 g, Cu 1 g, Co 0.25 g, I 0.25 g, Se 0.1 g, vitamin E 5 mg, vitamin A 3500000 IU, vitamin D3 750000 IU.
[2] Contained 50% α-tocopherol.
C = Control (concentrate); L = added linseed; CLA = added CLA; L+CLA = added linseed + CLA.

Table 2. Fatty acid profiles of the experimental diets [a].

Fatty Acid	C	L	CLA	L+CLA	SEM	*P*-value
C12:0	0.17	0.30	0.23	0.20	0.058	0.482
C14:0	0.85^{a}	0.30^{b}	0.66^{a}	0.25^{b}	0.065	<0.001
C15:0	0.05	0.04	0.05	0.05	0.004	0.360
C16:0	36.46^{a}	17.22^{c}	26.08^{b}	11.85^{c}	1.972	<0.001
C16:1c9	0.16^{a}	0.15^{a}	0.13^{a}	0.09^{b}	0.007	<0.001
C18:0	4.33^{b}	3.23^{b}	14.27^{a}	16.18^{a}	0.966	<0.001
C18:1c9	27.96^{a}	20.73^{c}	23.58^{b}	15.07^{d}	0.597	<0.001
C18:2n6c9c12	27.10^{b}	34.84^{a}	26.41^{b}	25.45^{b}	1.692	0.007
C18:3n6	0.02^{b}	0.09^{a}	0.01^{b}	0.10^{a}	0.004	<0.001
C20:0	0.40	0.29	0.37	0.31	0.028	0.076
C18:3n3c9,c12,c15	2.03^{b}	22.24^{a}	1.91^{b}	23.47^{a}	1.127	<0.001
9c11tCLA	0.00^{b}	0.00^{b}	2.46^{a}	2.91^{a}	0.153	<0.001
10t12cCLA	0.00^{b}	0.00^{b}	0.11^{a}	0.10^{a}	0.012	<0.001
9c11cCLA	0.17^{a}	0.17^{a}	0.09^{b}	0.06^{b}	0.012	<0,001
9t11tCLA	0.00^{b}	0.00^{b}	0.59^{a}	0.34^{ab}	0.104	0.004
C20:1c11	0.00^{b}	0.00^{b}	2.65^{a}	3.08^{a}	0.152	<0.001
C20:2c11,c14	0.02^{bc}	0.04^{a}	0.02^{c}	0.03^{ab}	0.003	<0.001
C22:0	0.16^{b}	0.19^{b}	0.22^{ab}	0.26^{a}	0.016	0.005
C20:4n6	0.00^{b}	0.03^{a}	0.00^{b}	0.03^{a}	0.002	<0.001
C22:1n9c13	0.00	0.00	0.01	0.02	0.005	0.061
C24:0	0.13	0.14	0.14	0.14	0.013	0.931
SFA	42.54^{a}	21.71^{b}	42.02^{a}	29.25^{b}	2.521	<0.001
MUFA	28.13^{a}	20.88^{b}	26.37^{a}	18.25^{c}	0.584	<0.001
PUFA	29.16^{b}	57.25^{a}	28.35^{b}	49.08^{a}	2.306	<0.001

[a] Based on 4 samples/concentrate. C = control (concentrate); L = 10% linseed; CLA = 2% CLA; L+CLA = 10% linseed and 2% CLA. Different letters in the same row indicate significant differences (P<0.05). SEM: Standard Error of Mean.

Table 3. Effect of linseed and/or CLA supplementation on polyunsaturated fatty acids (g/100 g total fatty acid) in young Holstein bulls[a].

	Intramuscular Tissue				Subcutaneous Tissue					*P*-value		
Fatty Acid	**C**	**L**	**CLA**	**L+CLA**	**C**	**L**	**CLA**	**L+CLA**	**SEM**	**Diet**	**Tissue**	**Diet x Tissue**
Σ PUFA	15.19	14.69	14.71	17.52	2.77	4.29	3.11	4.84	0.798	0.018	0.000	0.486
Σ*n6*	14.33	11.95	13.66	14.17	2.42	3.05	2.54	3.19	0.715	0.403	0.000	0.194
Σ*n3*	0.64 ^{b}	2.39 ^{a}	0.69 ^{b}	2.82 ^{a}	0.10 ^{c}	0.79 ^{b}	0.12 ^{c}	0.90 ^{b}	0.103	0.000	0.000	0.000
ΣCLA	0.29 ^{d}	0.42 ^{c}	0.42 ^{cd}	0.61 ^{b}	0.33 ^{cd}	0.56 ^{b}	0.59 ^{b}	0.87 ^{a}	0.031	0.000	0.000	0.006
C18:2n6t9t12	0.30 ^{d}	0.68 ^{b}	0.31 ^{d}	0.64 ^{b}	0.40 ^{cd}	0.99 ^{a}	0.43 ^{c}	0.95 ^{a}	0.027	0.000	0.000	0.000
C18:2n6c9c12, (LA)	10.16	8.37	9.72	10.24	1.85	1.86	1.89	2.02	0.528	0.223	0.000	0.300
CLA9c11t (RA)	0.12 ^{d}	0.24 ^{c}	0.23 ^{c}	0.38 ^{b}	0.18 ^{cd}	0.36 ^{b}	0.36 ^{b}	0.58 ^{a}	0.024	0.000	0.000	0.038
CLA10t12c	0.07 ^{d}	0.07 ^{d}	0.07 ^{d}	0.08 ^{cd}	0.08 ^{d}	0.10 ^{bc}	0.13 ^{a}	0.12 ^{ab}	0.006	0.000	0.000	0.000
CLA9c11c	0.05	0.03	0.05	0.03	0.03	0.03	0.03	0.04	0.006	0.098	0.179	0.138
CLA9t11t	0.05	0.09	0.07	0.12	0.03	0.07	0.06	0.13	0.006	0.000	0.026	0.092
C18:3n3 (ALA)	0.26 ^{c}	1.69 ^{a}	0.31 ^{c}	2.00 ^{a}	0.10 ^{c}	0.79 ^{b}	0.12 ^{c}	0.90 ^{b}	0.073	0.000	0.000	0.000
C18:3n6	0.08	0.08	0.08	0.08	0.03	0.04	0.03	0.04	0.004	0.101	0.000	0.152
C20:3n6	0.62 ^{a}	0.40 ^{c}	0.58 ^{ab}	0.44 ^{bc}	0.03 ^{d}	0.02 ^{d}	0.03 ^{d}	0.02 ^{d}	0.034	0.001	0.000	0.006
C20:4n6	2.73	2.13	2.57	2.44	0.04	0.04	0.04	0.05	0.170	0.371	0.000	0.343
C20:5n3 (EPA)	0.10 ^{b}	0.24 ^{a}	0.10 ^{b}	0.30 ^{a}	ND	ND	ND	ND	0.023	0.000	_	_
C22:4n6	0.38 ^{a}	0.24 ^{b}	0.35 ^{a}	0.25 ^{b}	ND	ND	ND	ND	0.025	0.000	_	_
C22:5n3 (DPA)	0.25 ^{b}	0.42 ^{a}	0.25 ^{b}	0.49 ^{a}	ND	ND	ND	ND	0.038	0.000	_	_
C22:6n3 (DHA)	0.03	0.03	0.03	0.03	ND	ND	ND	ND	0.003	0.800	_	_

[a] Based on 12 animals/dietary treatment. C = control (concentrate); L = 10% linseed; CLA = 2% CLA; L+CLA = 10% linseed and 2% CLA. SC = Subcutaneous; IM = Intramuscular. Different letters in the same row indicate significant differences ($P<0.05$). SEM: Standard Error of Mean.

ND = not detected.
Σ*n-6*: sum of C18:2n6t9t12, C18:2n6c9c12, CLA10t12c, C18:3n6, C20:3n6, C20:4n6 and C22:4n6
Σ*n-3*: sum of C18:3n3, C20:5n3, C22:5n3 and C22:6n3
ΣCLA: sum of CLA9c11t, CLA10t12c, CLA9c11c and CLA9t11t

Table 4. Effect of linseed and/or CLA supplementation on monounsaturated fatty acids (g/100 g total fatty acid) in young Holstein bulls[a].

	Intramuscular Tissue				**Subcutaneous Tissue**					***P*-value**		
Fatty Acid	**C**	**L**	**CLA**	**L+CLA**	**C**	**L**	**CLA**	**L+CLA**	**SEM**	**Diet**	**Tissue**	**Diet x Tissue**
Σ MUFA	35.48	38.36	36.70	36.18	44.40	46.33	45.27	45.03	0.932	0.077	0.000	0.956
C14:1c9	0.42	0.46	0.40	0.34	1.14	1.22	1.02	0.84	0.075	0.007	0.000	0.331
C15:1	0.02	0.01	0.01	0.01	0.02	0.02	0.02	0.02	0.001	0.012	0.000	0.489
C16:1t9	0.16	0.12	0.12	0.17	0.34	0.24	0.28	0.27	0.021	0.009	0.000	0.145
C16:1c9	2.62	2.53	2.43	2.10	4.85	4.67	4.19	3.50	0.200	0.000	0.000	0.153
C17:1c10	0.46	0.54	0.50	0.49	0.59	0.73	0.64	0.64	0.025	0.000	0.000	0.696
C18.1t9	0.25	0.29	0.24	0.41	0.36	0.35	0.37	0.46	0.027	0.000	0.000	0.456
C18:1t11 (VA)	2.40	2.98	2.48	4.19	3.51	3.83	4.08	5.90	0.337	0.000	0.000	0.527
C18:1c9	27.16	29.47	28.50	26.63	31.89	33.49	32.87	31.80	0.805	0.021	0.000	0.902
C18:1c11	1.88	1.82	1.88	1.73	1.54	1.61	1.58	1.39	0.053	0.005	0.000	0.522
C20:1c11	0.12	0.13	0.14	0.12	0.15	0.16	0.20	0.21	0.010	0.002	0.000	0.056

[a] Based on 12 animals/dietary treatment. C = control (concentrate); L = 10% linseed; CLA = 2% CLA; L+CLA = 10% linseed and 2% CLA. SC = Subcutaneous; IM = Intramuscular. Different letters in the same row indicate significant differences ($P<0.05$). SEM: Standard Error of Mean.

Table 5. Effect of linseed and/or CLA supplementation on saturated fatty acids (g/100 g total fatty acid) in young Holstein bulls[a].

	Intramuscular Tissue				Subcutaneous Tissue					*P*-value		
Fatty Acid	**C**	**L**	**CLA**	**L+CLA**	**C**	**L**	**CLA**	**L+CLA**	**SEM**	**Diet**	**Tissue**	**Diet x Tissue**
Σ SFA	49.33	46.95	48.59	46.30	52.83	49.39	51.62	50.12	0.833	0.001	0.001	0.852
C12:0	0.09	0.09	0.08	0.08	0.11	0.11	0.09	0.10	0.005	0.060	0.000	0.783
C14:0	2.49	2.74	2.40	2.41	4.01	4.22	3.85	4.12	0.154	0.149	0.000	0.835
isoC14:0	0.02	0.02	0.02	0.02	0.02	0.03	0.03	0.03	0.003	0.323	0.000	0.837
C15:0	0.41	0.43	0.40	0.42	0.55	0.58	0.56	0.60	0.027	0.563	0.000	0.912
isoC15:0	0.07	0.09	0.08	0.09	0.15	0.17	0.17	0.18	0.009	0.043	0.000	0.952
anteisoC15:0	0.09	0.13	0.11	0.13	0.20	0.29	0.25	0.30	0.014	0.000	0.000	0.224
C16:0	26.78	24.38	25.57	23.04	30.65	27.37	28.24	26.06	0.436	0.000	0.000	0.562
isoC16:0	0.08	0.10	0.09	0.09	0.11	0.12	0.13	0.13	0.007	0.025	0.000	0.779
anteisoC16:0	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.001	0.119	0.753	0.969
C17:0	0.69	0.79	0.74	0.80	0.78	0.84	0.87	0.91	0.031	0.002	0.000	0.521
isoC17:0	0.39	0.42	0.41	0.46	0.40	0.52	0.43	0.53	0.018	0.000	0.000	0.059
anteisoC17:0	0.33	0.45	0.37	0.44	0.59	0.74	0.63	0.66	0.018	0.000	0.000	0.224
C18:0	17.74	17.20	18.19	18.17	15.15	14.26	16.25	16.37	0.724	0.114	0.000	0.842
C20:0	0.13 [a]	0.11 [ab]	0.12 [ab]	0.13 [ab]	0.10 [c]	0.12 [ab]	0.11 [bc]	0.12 [ab]	0.004	0.058	0.000	0.000

[a] Based on 12 animals/dietary treatment. C = control (concentrate); L = 10% linseed; CLA = 2% CLA; L+CLA = 10% linseed and 2% CLA. SC = Subcutaneous; IM = Intramuscular. Different letters in the same row indicate significant differences ($P<0.05$). SEM: Standard Error of Mean.

Table 6. Effect of linseed and/or CLA supplementation on relevant nutritional ratios (g/100 g total fatty acid) in young Holstein bulls[a].

	Intramuscular Tissue				Subcutaneous Tissue					*P*-value		
Fatty Acid	**C**	**L**	**CLA**	**L+CLA**	**C**	**L**	**CLA**	**L+CLA**	**SEM**	**Diet**	**Tissue**	**Diet x Tissue**
ΣMUFA/ΣSFA	0.72	0.82	0.76	0.78	0.84	0.95	0.88	0.91	0.029	0.008	0.000	0.999
ΣPUFA/ΣSFA	0.31	0.32	0.31	0.38	0.05	0.09	0.06	0.10	0.019	0.007	0.000	0.482
Σ*n-6*/Σ*n-3*	22.26 ^{ab}	4.95 ^{d}	19.67 ^{c}	5.02 ^{d}	24.06 ^{a}	4.09 ^{d}	21.52 ^{bc}	3.71 ^{d}	0.490	0.000	0.295	0.001

[a] Based on 12 animals/dietary treatment. C = control (concentrate); L = 10% linseed; CLA = 2% CLA; L+CLA = 10% linseed and 2% CLA. SC = Subcutaneous; IM = Intramuscular. Different letters in the same row indicate significant differences ($P<0.05$). SEM: Standard Error of Mean.

SFA: Saturated fatty acids; MUFA: Monounsaturated fatty acids; PUFA: Polyunsaturated fatty acids

Σ*n-6*: sum of C18:2n6t9t12, C18:2n6c9c12, CLA10t12c, C18:3n6, C20:3n6, C20:4n6 and C22:4n6

Σ*n-3*: sum of C18:3n3, C20:5n3, C22:5n3 and C22:6n3

Table 7. Effect of linseed and/or CLA supplementation on the fatty acids (mg FA/100 g muscle) in the intramuscular depot from young Holstein bulls [a].

	C	L	CLA	L+CLA	SEM	*P*-value
ΣFA	1590.98	2146.66	1746.32	1911.97	202.047	0.260
ΣSFA	532.71	581.62	551.10	546.36	55.314	0.936
ΣMUFA	873.13	1374.77	1008.04	1148.48	153.656	0.136
ΣPUFA	185.14	190.28	187.18	217.13	13.068	0.284
Σ*n6*	169.03	154.06	168.17	174.65	10.952	0.593
Σ*n3*	11.83 [b]	29.81 [a]	12.51 [b]	33.59 [a]	1.837	0.000
ΣCLA	5.23 [b]	7.30 [ab]	7.43 [ab]	9.86 [a]	0.865	0.006
C16:0	285.88	300.34	287.63	271.46	30.094	0.926
C18:0	168.09	184.62	180.25	184.92	16.071	0.868
C18:1t11 (VA)	14.19 [b]	21.03 [ab]	14.97 [b]	26.31 [a]	2.724	0.009
C18:1c9	725.80	1203.22	856.82	984.51	143.514	0.127
C18:2n6c9c12 (LA)	106.71	95.07	106.36	111.46	7.116	0.422
CLA9c11t (RA)	2.29 [b]	3.90 [b]	3.83 [b]	5.93 [a]	0.513	0.000
CLA10t12c	0.95	0.90	0.93	0.97	0.108	0.970
CLA9c10c	1.07	0.76	1.32	0.75	0.219	0.210
CLA9t11t	0.93 [c]	1.75 [ab]	1.34 [bc]	2.21 [a]	0.191	0.000
C18:3n3 (ALA)	1.84 [b]	12.28 [a]	2.08 [b]	13.50 [a]	0.688	0.000
C20:5n3 (EPA)	2.39 [b]	4.91 [a]	2.54 [b]	5.91 [a]	0.456	0.000
C22:5n3 (DPA)	6.62 [b]	11.78 [a]	6.91 [b]	13.26 [a]	0.911	0.000
C22:6n3 (DHA)	0.97	0.84	0.98	0.92	0.121	0.834

[a] Based on 12 animals/dietary treatment. C = control (concentrate); L = 10% linseed; CLA = 2% CLA; L+CLA = 10% linseed and 2% CLA. Different letters in the same row indicate significant differences ($P<0.05$). SEM: Standard Error of Mean.
SFA: Saturated fatty acids; MUFA: Monounsaturated fatty acids; PUFA: Polyunsaturated fatty acids

Σ*n-6*: sum of C18:2n6t9t12, C18:2n6c9c12, CLA10t12c, C18:3n6, C20:3n6, C20:4n6 and C22:4n6
Σ*n-3*: sum of C18:3n3, C20:5n3, C22:5n3 and C22:6n3
ΣCLA: sum of CLA9c11t, CLA10t12c, CLA9c11c and CLA9t11t

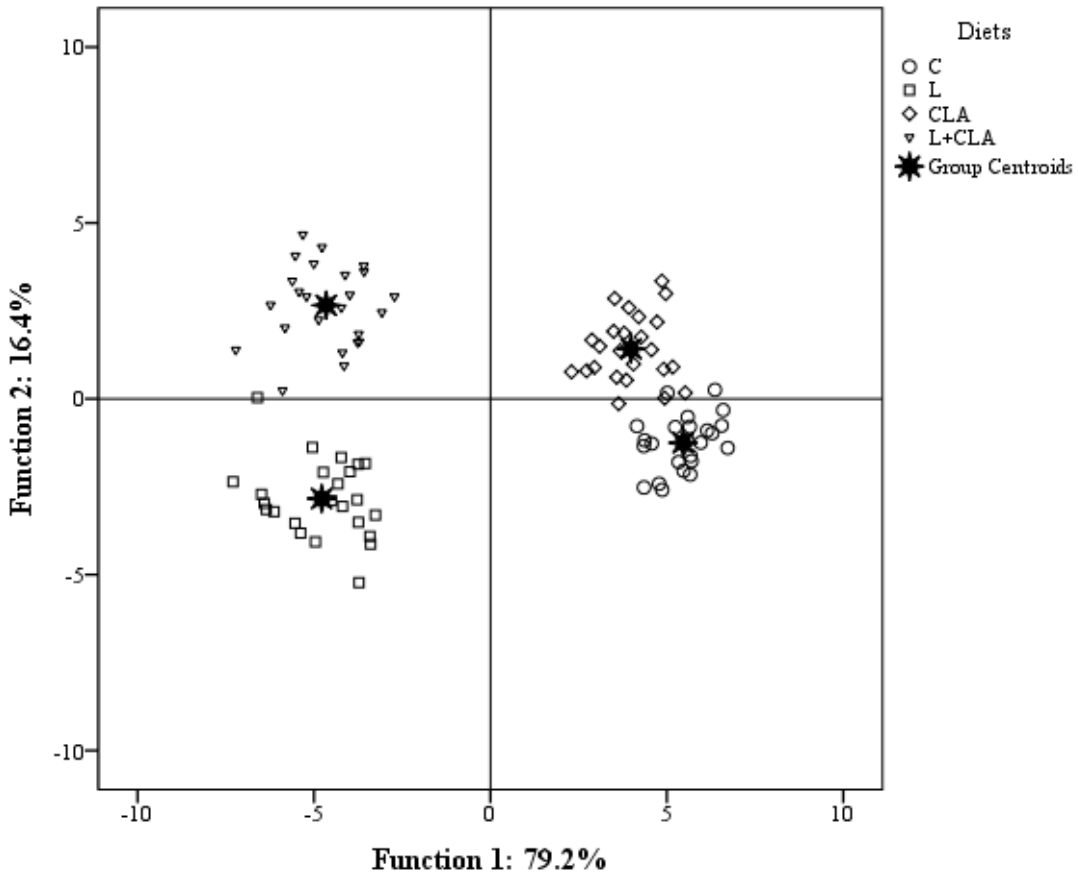

Figure 1. Spatial location of samples from young Holstein bulls according to stepwise discriminate analysis including fatty acids of intramuscular and subcutaneous tissues. Diets: C = control (concentrate); L = 10% linseed; CLA = 2% CLA; L+CLA = 10% linseed and 2% CLA.

5. Consumidores

Relative importance of cues underlying Spanish consumers' beef choice and segmentation, and sensory acceptability of beef enriched with *n*-3 and CLA fatty acids

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1. Abstract

The Choice experiments technique was used to estimate the relative importance of the main attributes of beef meat; origin (locally produced, not locally produced), animal diet (conventional, enriched with *n-3*, with CLA, or with *n-3* plus CLA), amount of visible fat (slight, moderate), meat colour (bright red, pale red), and meat price (6.6, 5.7, 4.8, 3.9 € per tray containing 0.3 kg of beef loin) in three Spanish cities (Barcelona, Pamplona, Zaragoza) with 322 individuals. Consumer segments with similar preference profiles of beef choices were identified using the Latent Class model. The sensory acceptability of beef enriched with *n-3* and/or CLA fatty acids was also evaluated. The most important cue driving the majority of consumers' beef purchase decisions was the amount of visible fat (36%) followed by price (25%), then colour (19%) and origin (17%), and finally animal diet (4%) which was not important. Most consumers preferred beef with a slight content of visible fat, medium-low price, locally produced and bright red colour. Segmentation analysis revealed three consumer profiles: 'health conscious consumers' with preference for lean beef (consumers: older, women, retired, housewives, greater food expenditure and more concerned with health); 'price-oriented consumers' with preference for lowest priced beef (consumers: younger, students, higher education, lower beef intake and purchase at supermarket); and 'quality-oriented consumers' with preference for beef with moderate amount of visible fat (consumers: men, from Barcelona). Consumers were willing to pay a premium of 1.21, 1.52 and 2.04€ over 14€/kg for CLA, *n-3* and *n-3* plus CLA enriched beef, respectively. The individual beef enrichment with *n-3* or CLA improved sensory acceptability scores, while the combined enrichment with *n-3* plus CLA offered no sensory advantages over

conventional beef. Marketing efforts for enriched beef would be expected to be more successful in Barcelona than Pamplona and Zaragoza, and 'health conscious consumers' would be more willing to pay and more likely to purchase enriched beef with beneficial fatty acids.

Keywords: Meat, *n-3*, CLA, consumers, choice experiments, random parameters logit model, latent class model.

1. Introduction

Consumers' inference of product quality at the point of purchase is based on available extrinsic and intrinsic cues that they believe reflect product quality (Banović, Grunert, Barreira & Fontes, 2010). Generally, extrinsic quality cues include price, product presentation, origin and brand, while important intrinsic quality cues for meat include the physiological characteristics of the product such as colour and visible fat (Troy & Kerry, 2010). Of all the cues consumers are exposed to, only those which are perceived and used will influence the expected quality and thus product evaluation (Grunert, 1997). Relevant cues have been identified in previous studies for beef (Bernués, Olaizola & Corcoran, 2003a; Mesías, Escribano, de Ledesma & Pulido, 2005; Olaizola Tolosana, Whebi & Manrique Persiva, 2005; Realini, Font i Furnols, Sañudo, Montossi, Oliver & Guerrero, 2013), but consumer meat quality perception is complex, difficult to define and dynamic (Troy et al., 2010). (Troy et al., 2010) indicated that it is essential for the meat industry to fully understand what these cues are and which are the most important as well as what can be done to maintain and enhance these cues in existing and new products. According to (Mennecke, Townsend, Hayes & Lonergan, 2007) consumers will be willing to pay more for those characteristics they value, as consumers are exposed to beef products with a greater variety of features and attributes that are preferred. Some of the main trends in consumer lifestyles with regard to meat when purchasing are related to increasing use of extrinsic cues in quality perception associated with increased awareness of the link between food and health, and consumers' interest in the origin and production of their food (Grunert, 2006). In the past food quality was more related to safety, sensory and shelf-life aspects of food products, but more

recently it is associated with nutrition, well-being and health (da Fonseca & Salay, 2008; Troy et al., 2010; Verbeke, Frewer, Scholderer & De Brabander, 2007).

Meat and meat products can be viewed as having a double mirror image with respect to composition and nutrition (Troy et al., 2010), partly due to conflicting findings from medical research. Meat and meat products are generally recognized as highly nutritious foods that provide valuable amounts of protein, fatty acids, vitamins, minerals and other bioactive compounds (Olmedilla-Alonso, Jiménez-Colmenero & Sánchez-Muniz, 2013), and consumers consider meat to be a healthy and important component of the diet (Verbeke, Pérez-Cueto, de Barcellos, Krystallis & Grunert, 2010). However, red meat and processed meat consumption have been associated with a number of unfavorable health conditions, such as cardiovascular disease (WHO, 2003), some types of cancers (Sato, Nakaya, Kuriyama, Nishino, Tsubono & Tsuji, 2006), and diabetes (Schulze, Manson, Willett & Hu, 2003) linked to the contribution of meat and meat products to the intake of fat, saturated fatty acids, cholesterol, salt, and other substances that may have negative health implications (Olmedilla-Alonso et al., 2013). According to (Verbeke et al., 2010), the increased transparency about the nutritional content of food products may also induce changes in consumer demand, and has already led producers to reformulate some meat products with lower fat or higher polyunsaturated fatty acid content. Because of their health implications, lipids are among the bioactive components that have received most attention, particularly with respect to the development of healthier meat products, and (Olmedilla-Alonso et al., 2013) supports that meat and meat products are excellent foods for delivering bioactive compounds without changing dietary

habits. Omega-3 (*n*-3) fatty acids play a major role in human health and are involved in the development of brain and retinal tissues and in the progression and prevention of human diseases, including heart disease and some cancers (Connor, 2000; Simopoulos, 1999). Conjugated linoleic acid (CLA) is a functional meat ingredient that has received special research attention. Naturally produced by ruminant animals, CLA has the potential to reduce the risk of cancer, cardiovascular diseases, diabetes, and obesity, as well as to boost the immune system (Peng, West & Wang, 2006).

Meat composition varies with respect to numerous factors and animal diet is the factor which can most easily be manipulated and which has one of the most profound effects on its composition (Troy et al., 2010). Thus, numerous animal feeding trials have been carried out in the attempt to increase the *n*-3 and the CLA content in beef (Olmedilla-Alonso et al., 2013). The most common animal feeding system in Spain is based on diets rich in concentrates with very limited content of forages leading to beef with a fatty acid composition excessively high in *n*-6 fatty acids. Nonetheless, it is possible to enrich the *n*-3 and CLA fatty acid content of meat by feeding animals linseed (source of *n-3*) and rumen protected CLA (direct source of CLA). However, modifying the type of fat in meat to obtain a fatty acid profile that better matches the current nutritional recommendations for a healthy diet may affect other properties of meat such as flavor, but consumers are not willing to compromise on taste of functional foods for eventual health benefits as indicated by many authors (Augustin, 2001; Cox, Koster & Russell, 2004; Gilbert, 2000; Verbeke, 2006). (Verbeke, 2006) stressed that monitoring taste emerges as an extremely critical factor for the future acceptance of functional foods, and food producers considering marketing a

functionally enriched alternative should be very particular in their research of consumer attitudes to the particular base-product and enrichment involved (Bech-Larsen & Grunert, 2003). Thus, consumer sensory acceptability of enriched beef with *n-3* and/or CLA should be evaluated, as limited data are available for beef enriched with either *n-3* or CLA, and no consumer sensory acceptability data are available for beef enriched with both *n-3* and CLA.

Other cues than animal diet have been identified as important criteria affecting consumers' perceptions about product quality including meat origin and price as extrinsic factors, and colour and visible fat as intrinsic factors. (Olaizola Tolosana et al., 2005) evaluated the importance of beef quality attributes in Spain, and indicated that animal feeding regime, origin/region of production, animal welfare, and slaughter conditions were considered most important by consumers. (Mesías et al., 2005) showed that product origin was the most important attribute for the choice of beef, followed by quality labeling, production system and price in a different region of Spain. More recently, (Realini et al., 2013) reported that country of origin was the most important factor in the beef choice of Spanish consumers, followed by animal feeding, with price being the least important attribute. The importance attached to location of production is of particular relevance to develop marketing strategies involving protected geographical indication and meat branding programs for the domestic and regional Spanish beef markets.

The price of meat is another important extrinsic cue that can affect consumer purchase decisions (Lange, Rousseau & Issanchou, 1998; Lockshin, Jarvis, d'Hauteville & Perrouty, 2006), especially when the product cannot be fully

evaluated prior to purchase as it is the case with meat. Identified intrinsic attributes used by consumers to infer meat quality are meat colour which is the first attribute evaluated as the indicator of quality and freshness (Faustman & Cassens, 1990; Glitsch, 2000), and the amount of visible fat that is used as a health (Issanchou, 1996) and palatability (Miller, 2002; Sánchez, Beriain & Carr, 2012) indicator at the point of purchase.

Purchase decisions are based on simultaneous evaluation of multiple product attributes, and consumers make certain tradeoffs with respect to those characteristics when selecting a product (Moskowitz, Beckley & Minkus-McKenna, 2004). Several alternatives are available to analyze consumer preferences (Kallas *et al*., 2011), and the choice experiment (CE) is one of the most relevant techniques due to its capacity to analyze preferences for 'complex goods' as it is the case of food products. The CE is a stated preference method which is based on the creation of hypothetical markets for the analyzed goods and services. It involves the characterization of the product through a series of descriptors (attributes and their levels) that can be combined following an experimental design to create different proposed scenarios of the product (alternatives). These scenarios differentiate the analyzed product in one or more attribute levels. Respondents are faced with several of these scenarios (choice sets) and are asked to select their preferred product, while implicitly making a trade-off between those attributes. Furthermore, they would be asked if they are willing to purchase their preferred product at different price levels. To predict consumers' preferences, the probability of choice that an individual *n* chooses the alternative *i* rather than the alternative *j* needs to be defined. Two models were used in this study,

the Random Parameters Logit model (RPL) and the Latent Class model (LC). The RPL model accommodates for the unobserved heterogeneity of the whole sample. This model introduces heterogeneity preference assuming that for each attribute level (X_k) there is a mean effect (β_k) and a standard deviation (φ_k). The LC model deals with the observed sources of heterogeneity. It allows exploring preference differences across individuals taking into account the probability of membership to a latent segment. In this model, the utility provided by alternative i to subject n is segment dependent (s).

The objectives of this study were: (i) to determine the relative importance of extrinsic (origin and price) and intrinsic (fat, colour, and enrichment with *n-3* and/or CLA fatty acids) cues underlying Spanish consumers' beef preferences by applying the choice experiments technique; (ii) to identify profiles of beef consumers with similar perceptions and intentions; (iii) to characterize these profiles according to their socio-economic features, attitudes and behavior; and (iv) to evaluate the sensory acceptability of beef enriched with *n-3* and/or CLA fatty acids.

2. Materials and Methods

The study was carried out with 322 individuals in three Spanish cities: Barcelona (n=100), Pamplona (n=109) and Zaragoza (n=113). Consumers were selected by means of a probabilistic sampling per quotas according to the national distribution by gender and age. The socio-demographic characterisation of the selected consumers is shown in Table 1.

2.1. Choice experiments analysis

Choice experiments analysis was used to determine the relative importance of five evaluated attributes and their levels in purchasing decisions of beef by consumers in three Spanish cities (Barcelona, Zaragoza, and Pamplona). The attributes evaluated in this study (animal diet, origin, colour, fat content and price) were chosen because of their importance in consumer purchasing decisions reported in the literature (Bello Acebrón & Calvo Dopico, 2000; Bernués et al., 2003a; Mesías et al., 2005; Olaizola Tolosana et al., 2005), as well as a focus group discussion comprised by university lecturers and researchers from the meat science and agro-food marketing fields.

Four levels were evaluated for the animal diet attribute which corresponded to the type of beef assessed in the sensory evaluation described in section 2.2 (CONV: conventional, OME3: enriched with omega-3, CLA: enriched with CLA, OME3CLA: enriched with omega-3 plus CLA). Beef is associated by many consumers with meat rich in saturated lipids and poor health, which has favoured research on altering the fatty acid composition of meat through changes in the animal diet in order to match more closely current nutritional recommendations for a healthy diet. Thus, it is possible to enrich the *n*-3 and CLA fatty acid content of meat through animal supplementation with linseed (source of *n*-3) and rumen protected CLA (source of CLA). Consumers were not given any information about the *n*-3 and CLA fatty acids or their role in human health. Origin had two levels which involved Barcelona, Zaragoza or Pamplona as 'locally produced' and other Spanish origin as 'not locally produced'. The relative preference for locally produced beef compared with other Spanish origin is important to develop marketing strategies for the domestic and regional Spanish beef markets. Consumers base their purchasing

choices on perceived quality and colour is the first attribute evaluated as the indicator of meat quality and freshness (Faustman et al., 1990; Glitsch, 2000). Thus, two colour levels were evaluated in the current study as 'pale red' or 'bright red'. The fat content of beef is also a key attribute and (Issanchou, 1996) indicated that consumers use visible fat as a health indicator at the point of purchase. Two fat levels of beef steaks were evaluated as 'moderate visible fat' or 'slight visible fat'. Finally, beef price was considered as another key attribute with four levels. The price vector was not determined by the actual price of the product, but rather by the unobserved demand curves and thus, was based on prior knowledge concerning the maximum willingness to pay (Mørkbak *et al*., 2010). In this context, a pilot study with 25 individuals was carried out, dealing with respondents' maximum willingness to pay for enriched beef meat using an open-ended valuation question. Finally, the price levels included in the choice sets were selected in order to cover the central 90% of the observed values across respondents. The selected price levels were defined in € per consumer unit (one tray containing one beef steak: 0.3 kg of loin) as follows: 6.6€ high, 5.7€ medium-high, 4.8€ medium-low and 3.9€ low meat price.

The combination of all the factors and their levels resulted in 144 (2^4x3^2) hypothetical products obtaining $(2^4x3^2)^3$ possible combinations or choice sets (three hypothetical products per choice set). An orthogonal fractional factorial design was used to reduce the number of choice sets from the initial possible combinations in the full design to only 16 choice sets. Thus, a factorial blocking arrangement was carried out obtaining 2 blocks, each with 8 choice sets presented to individual respondents so that the number of profiles would be low enough to be easily handled by consumers. An example of a single choice set composed by 3 hypothetical products is shown in

Figure 1 and each consumer evaluated a total of 8 choice sets. Consumers were asked to carefully evaluate each choice set, choose one of the three available products and subsequently indicate if they would purchase the chosen product following the dual response choice experiment design proposed by (Kallas & Gil, 2012). Pictures of beef loin steaks were computed manipulated using Photo Editor to obtain the evaluated levels of colour and fat content as previously done successfully by other authors (Fortomaris, Arsenos, Georgiadis, Banos, Stamataris & Zygoyiannis, 2006; Papanagiotou, Tzimitra-Kalogianni & Melfou, 2013).

2.2. The relative importance of the attributes: the RPL model

Following the choice experiments literature (Hensher, Rose & Greene, 2005) the probability that an individual *n* will choose the product i (P_{in}) among other products ($j = 1$ to J) of a set of them (C) is formulated as follows:

$$P_{in} = \frac{e^{\mu V_{in}}}{\sum_{j=1}^{j=J} e^{\mu V_{jn}}} \qquad \forall i \in C$$

where μ is a scale parameter which is inversely proportional to the standard deviation of the error terms. Within this model, the V_{in} must be defined. We followed the definition of the Random Parameter Logit model (RPL) since it relaxes the restrictive property of the Independence of Irrelevant Alternatives (IIA) of the traditional choice models. In this model the utility function assumes that for each attribute level (X_k) there is a mean effect (β_k) and a standard deviation (φ_k): $V_{in} = \beta_i + \sum_k \beta_k X_{ki} + \sum_k \varphi_k X_{ki}$. The main innovation of the RPL (known also as the mixed logit model) is the assumption that the functional form and the coefficients of

utility are common across respondents, but that the parameters vary across the individuals. Correspondingly, the utility function can be obtained for each participant and thus be compared with the individual sensory results. Finally, for the calculation of the relative importance, we used the marginal utilities (β_k) attached to the levels of the attributes obtained from the RPL model. Thus, the ratio of particular attribute utility to the sum of all attributes' utilities is used to reveal the relative importance of a particular attribute by the following equation (Smith, 2005):

$$O_k = \frac{\left(\max u_k - \min u_k\right)}{\sum_{k=1}^{K}\left(\max u_k - \min u_k\right)}$$

where O_k is the relative importance of the product attribute; $\max u_k$ is the utility of the attribute's most preferred level and $\min u_k$ is the utility of the least preferred level.

2.3. Consumer segmentation by the Latent Class Model

To identify the latent segments (clusters) inherent to a sample on the basis of the individuals' utilities of the attributes and levels, we used the Latent Class model. In this model the utility that consumer *n*, who belongs to a particular segment s, derives from choosing product i can be written as: $V_{in/s} = \beta_{is} + \sum_k \beta_{ks} X_{ki}$, where β_{ks} is a segment-specific vector of coefficients. The differences in β_{ks} enables this approach to capture the heterogeneity in attribute preferences across segments. When applying the LC model, researchers must first determine the number of the latent class. A very common criteria used is the Akaike Information Criteria (AIC). Thus, the numbers of classes that minimize this criterion suggest the preferred model. However, when different criteria (pseudo-R^2, AIC) indicate different preferred numbers of segments,

the selection must also account for significance of parameter estimates and be tempered by the analyst's own judgment on the meaning of the parameter signs. In this study, we estimated three classes for each sample as the best option that better explains respondents' heterogeneity. It's worth mentioning that the LC goal of classification into *T* homogeneous groups of the sample is identical to that of the traditional cluster analysis. However, in contrast to an *ad hoc* measure of distance used in the traditional cluster analysis to define homogeneity, LC model defines homogeneity in terms of probabilities to belonging to the different estimated groups.

2.4. Beef sampling procedure for sensory analysis

Spanish beef was obtained for sensory analysis from forty-eight Friesian entire males fed with one of four dietary treatments. All animal diets had similar composition but differed in the content of whole linseed and conjugated linoleic acid (CLA): CONV (conventional commercial ration, 0% linseed and 0% CLA), OME3 (conventional ration enriched with omega-3 fatty acids through the addition of 10% linseed), CLA (conventional ration enriched with CLA through the addition of 2% CLA), and OME3CLA (conventional ration enriched with omega-3 and CLA fatty acids through the addition of 10% linseed plus 2% CLA). Animals were slaughtered with an average live weight of 458.4±16.6 kg at an EU-licensed commercial abattoir following standard procedures. Animal productive performance and carcass characteristics of these animals were reported by (Albertí et al., 2013) and meat quality traits by Barahona et al. (2013). The left *Longissimus dorsi* muscle was removed from each carcass at 24 h post-mortem and cut into 2-cm thick steaks from the 10th thoracic rib in the caudal direction. Samples were vacuum packaged, aged at

2±2ºC during 7 days, frozen at -18±2ºC and transported to Barcelona, Zaragoza and Pamplona for consumer sensory evaluation. Before the sensory analysis, samples were thawed at 2±2ºC for 24 h and cooked in a double hot-plate grill pre-heated to 200ºC until final internal temperature reached 71ºC that was determined using individual thermocouples inserted into the geometric centre of the meat. Steaks were trimmed of external fat and connective tissue, cut into 2x2x2 cm samples, wrapped individually in coded aluminium foil and kept warm in a heater until tasting. Beef from one animal was evaluated by 10 consumers from each of the 3 Spanish cities, and beef from 12 animals per dietary treatment was evaluated by all consumers in each city.

2.5. Consumer sensory evaluation

Ten sensory sessions were conducted in Barcelona, Zaragoza and Pamplona with approximately ten consumers per session and beef samples from four animals were evaluated per session. Consumers evaluated in a blind condition the acceptability of four different grilled *Longissimus dorsi* samples of beef (4 diets: CONV, OME3, CLA, and OME3CLA) under white lights in the order printed on the recording sheet which was established to avoid the effect of sample order presentation, first-order or carry-over effects (Macfie, Bratchell, Greenhoff & Vallis, 1989). Consumers ate unsalted toasted bread and drank mineral water to rinse their palate between samples. Each consumer rated overall acceptability using a 9-point category scale (1‘dislike extremely’, 2‘dislike very much’, 3‘dislike moderately’, 4‘dislike slightly’, 5 ‘neither like nor dislike’ 6‘like slightly’, 7‘like moderately’, 8‘like very much’, 9‘like extremely’).

2.6. Attitudes and behaviour of consumers

Consumer attitudes and behaviour information were obtained from a closed-type survey comprised of questions with different types of measurement scales in the responses as indicated in Table 2.

2.7. Statistical analysis

Data analysis was conducted for each city individually (Barcelona: n=100, Zaragoza: n=113, Pamplona: n=109), and globally for all countries (n=322). Overall acceptability data were analyzed using the MIXED procedure of SAS (SAS Inst. Inc., Cary, NC), and mean separation was carried out using the Tukey's test. The statistical model for each individual city included dietary treatment as a fixed effect, consumer as random, and session as a block effect. The statistical model for all cities included dietary treatment and city as fixed effects, consumer within city as random, and session within city as a block effect. In addition, for each cluster obtained previously by choice analysis, acceptability mean values were calculated and analyzed using the same statistical model as for all cities.

3. Results

3.1. Consumers' beef choice by city: Barcelona, Zaragoza and Pamplona

The relative importance of each factor and utilities for each level of the factors are presented for consumers from Barcelona, Zaragoza and Pamplona in Table 3. Fat content, beef colour and price were important factors while beef origin and animal diet were non significant factors in the choice of beef by consumers from

Barcelona. Positive and significant utility values showed a preference for bright red beef with low levels of visible fat and medium priced. Consumers from Zaragoza assigned higher and similar importance to the fat content and price of beef, followed by origin of production and animal diet with no importance in the choice of beef for meat colour. Consumers from Zaragoza preferred beef produced locally with low levels of visible fat while beef enriched with CLA and highest meat price were the least preferred. The no option coefficient was positive and significant implying that in some cases respondents from Zaragoza did not choose any of the evaluated beef choices. Similar importance was given by consumers from Pamplona to origin, fat content, colour and price of beef while animal diet was not an important factor in their beef choice. Consumers from Pamplona preferred locally produced beef with low levels of visible fat, bright red colour and medium-low priced, while beef of the highest price was least preferred. Similarly to consumers from Zaragoza, consumers from Pamplona did not select in some cases any of the given beef choices.

3.2. Consumers' beef choice for all cities and consumer clusters

The relative importance of each factor and utilities for each level of the factors are presented for all consumers and for each consumer cluster in Table 4. The fat content of beef was the most important attribute assessed for beef purchase intention by all evaluated consumers followed by beef price, then beef origin and colour and finally animal diet which was not considered important. Positive and significant utilities indicate a preference for beef produced locally, with low levels of visible fat, bright red colour and medium-low priced, while beef of the highest price was least preferred. In some cases consumers did not choose any of the evaluated

beef choices. Consumer cluster 1 assigned highest importance to the fat content of beef, lower to origin of production, and lowest and similar importance to animal diet, beef colour and meat price. Consumers in cluster 1 preferred beef produced locally, with low levels of visible fat, and bright red colour and enriched with omega-3, while beef of lowest price was the least preferred. The no option coefficient was negative and significant implying that respondents would prefer to move away from purchasing none of the proposed products and accept enriched beef even if they would have to pay more for it. Fat content and price of beef were the two most important factors for the choice of beef for consumers in cluster 2, followed by animal diet and beef colour and finally origin of production. Consumers in cluster 2 preferred locally produced beef with low levels of visible fat, bright red colour, from conventionally fed animals and lower meat prices, while higher meat prices and beef enriched with both omega-3 and CLA were least preferred. In some cases consumers from cluster 2 did not choose any of the evaluated beef choices. Fat content was the most important beef attribute for purchasing choices by consumers in cluster 3, then beef price followed by animal diet and beef colour with no importance in the choice of beef for origin of production. Consumers in cluster 3 preferred beef with moderate visible fat, bright red colour, from animals fed conventional diets and medium meat prices, while beef enriched with CLA and highest and lowest meat prices were least preferred. Respondents in cluster 3 selected one of the evaluated beef choices in all cases even if they would have to pay more for their choice.

3.3. Sensory acceptability of beef enriched with n-3 and/or CLA

The overall consumer acceptability of beef enriched with *n-3* and/or CLA is shown Table 5 by city, for all cities and for consumer clusters identified based on consumers' beef choices (data from Table 3). Beef acceptability did not differ ($P > 0.05$) among dietary treatments when evaluated by consumers from Barcelona. However, overall acceptability was higher for beef enriched with OME3 and CLA than CON and OME3CLA when assessed by consumers from Zaragoza, Pamplona or pooled consumers across cities. Consumers from cluster 1 assigned higher ($P<0.05$) acceptability scores for OME3 than CON and OME3CLA, and CLA scores tended to be higher ($P<0.10$) than OME3CLA. Overall sensory acceptability of OME3 was higher than OME3CLA for consumers in cluster 2. Finally, beef acceptability by consumers in cluster 3 tended to be higher ($P<0.10$) for OME3 and CLA than CON.

3.4. Demographics, attitudes and behavior of consumers and their clusters

The socio-demographic characterisation of the selected consumers is shown in Table 6 for all consumers and their clusters identified based on consumers' beef choices (data from Table 3). The percentage of women was higher in cluster 1 while the percentage of men was higher in cluster 3 with intermediate percentages for consumers in cluster 2. The age of consumers was higher in cluster 1, intermediate in cluster 3 and lower in cluster 2. Cluster 1 and 3 showed higher percentage of consumers with primary studies or less and lower percentage with university studies than consumers in cluster 2. There were no differences ($P>0.05$) in the income level of consumers among clusters. Clusters 2 and 3 showed a higher percentage of consumers as 'students', clusters 1 and 3 a higher percentage of 'retired' consumers

and cluster 1 a higher percentage of 'housewife' consumers than the other clusters. Cluster 3 had higher percentage of consumers from Barcelona, cluster 2 from Pamplona and Zaragoza, while consumers in cluster 1 were balanced across cities.

Consumer attitudes and behaviour for all consumers and their clusters identified based on consumers' beef choices (data from Table 3) are shown in Table 7. Frequency of beef and lamb consumption was higher for consumers in clusters 1 and 3 than cluster 2, while pork and chicken consumption was similar among clusters. The percentage of consumers that buy fresh beef at the supermarket was higher in clusters 2 and 3 than cluster 1, and was higher at the traditional market in clusters 1 and 3 than cluster 2. There was a higher percentage of consumers in the lower level of food expenditure in clusters 2 and 3, and a higher percentage in the upper level in cluster 1 compared with the other clusters. A higher percentage of consumers in cluster 1 indicated to have high cholesterol, assigned higher scores to check their health periodically and to be used to purchase enriched food than consumers in cluster 2 with consumers from cluster 3 being intermediate. Consumers in cluster 2 indicated longer time since last blood analysis than consumers in clusters 3 and 1. Consumers in cluster 1 were willing to pay more for enriched beef with omega-3 and with both omega-3 and CLA than consumers in the other clusters. Finally, the probability to purchase different types of enriched meat with omega-3 and/or CLA was higher for consumers in cluster 1, followed by consumers in cluster 3 with lower probability for consumers in cluster 2, except for enriched pork which was similar for consumers in clusters 2 and 3.

4. Discussion

4.1 Choice experiment analysis

The most important cue driving the majority of consumers' beef purchase decisions was the amount of visible fat followed by beef price, then beef origin and colour, and finally animal diet which was not considered important. Meat quality traits such as colour, freshness and fat of beef can influence consumer-purchasing decisions and beef expectation increases with an attractive appearance and freshness and decreases with the amount of visible fat (Bello Acebrón et al., 2000; Steenkamp & VanTrijp, 1996). There are two key roles associated with beef fat, its potential impact on human health depending on the amount and type of fat and its impact on meat sensory quality. According to (Issanchou, 1996), consumers use visible fat (external and internal) as a health indicator at the point of purchase and they associate high fat content with poor health. On the other hand, it has been shown that increasing levels of marbling in meat are linked to increased palatability (Miller, 2002; Sánchez et al., 2012). However, the improvements in palatability with increasing fat percentage are not equal across all fat levels and the fat content preference is market specific. It has been shown that beef marbling is an important positive expectation generator in several markets because there are consumers who relate marbling with eating quality (Egan, Ferguson & Thompson, 2001). Consumers from some Asian countries (Japan, Taiwan and Korea) prefer raw beef with a moderate amount of marbling (Egan et al., 2001; Ngapo, Martin & Dransfield, 2007). However, several studies have shown that consumers prefer lean steaks with a minimal amount of marbling (Bello Acebrón et al., 2000; Grunert, 1997; Killinger, Calkins, Urnberger, Feuz & Eskridge, 2004; Savell et al., 1989), and according to (Bredahl, 2004; Killinger et al., 2004), consumers that prefer lean meat consider leanness as an important selection criteria

and they have a clear negative perception of fat in meat. Results from the current study showed that the amount of visible fat in beef is the most important attribute for beef purchasing decisions with a clear preference for slight over moderate fat content regardless of the city. The limited consumers' knowledge about the contribution of marbling to beef eating quality could easily lead to dissatisfaction when consumer expectations at purchase do not match the sensory experience at the moment of consumption (Morales, Aguiar, Subiabre & Realini, 2013). Finding an optimum fat level to achieve a visually acceptable marbling that does not compromise sensory quality in each market as well as consumer education about the link between fat and palatability in meat will contribute to reduce the gap between expected and experienced quality of beef steaks. (Morales et al., 2013) showed that information about marbling level and production system generated positive expectations and increased acceptability ratings for beef with low marbling levels and beef from grazing animals.

Meat price was the second most important factor accounting for consumers' beef purchasing decisions and the medium-low price of beef was preferred by most consumers. Results showed that the utility decreased as the meat price increased over medium-low as reported in previous studies by (Font i Furnols et al., 2011; Mesías et al., 2005; Realini et al., 2013) for beef and lamb. Meat price has been found to be an indicator of quality and safety with a higher price linked to higher beef quality (Banović et al., 2010; Bello Acebrón et al., 2000) and a lower price associated with unsafe meat (Verbeke et al., 2010). The preference for medium-low price over the cheapest beef may be explained by the negative perception of consumers from meat

that is too cheap and the positive relationship between meat quality and price. In contrast to the results from the present study, some authors have found that the price of meat was the least important factor accounting for consumers' beef purchasing decisions (refs). According to (Bello Acebrón et al., 2000), price appears as a relevant cue when consumers lack information about intrinsic quality cues or when it is the only available cue. This was not the case in the present study where other cues including intrinsic quality cues were available for consumer choices. (McCarthy, de Boer, O'Reilly & Cotter, 2003) highlighted that while price is important it alone cannot explain changes in meat consumption, and indicated that the percent contribution of price and income to change in beef and veal consumption had reduced. However, the current compromised economic situation in Spain may result in an increased relative importance of the price of meat as an extrinsic cue for consumer purchasing decisions for beef.

Following visible fat content and meat price, beef origin and meat colour were considered more important attributes than animal feeding by most evaluated consumers except by respondents from Barcelona. (Grunert, 2006) highlighted the increasing importance of extrinsic cues in consumers' quality perception of meat including the origin and production of their food. Geographical origin of meat has been pointed out as an indicator of meat safety (Cowan, 1998; Latouche, Rainelli & Vermersch, 1998; Verbeke et al., 2010) and has been linked to the value of 'locality', 'consumer sense of belonging' (Bernués et al., 2003a; De Cicco, Van der Lans & Loseby, 2001), social acceptance and support to local meat producers (Papanagiotou et al., 2013). Moreover, previous research has found that consumers were willing to

pay a premium for locally produced meats (Jekanowski, Williams & Schiek, 2000; McGarry-Wolf & Thulin., 2000; Thilmany, Grannis & Sparling, 2003; Umberger, Feuz, Calkins & Sitz, 2003). Origin is a significant attribute when purchasing beef in many countries with consumer preference for domestic beef (Alfnes, 2004; Bernués, Olaizola & Corcoran, 2003b; Henson & Northen, 2000; Mesías et al., 2005; Quagrainie, Unterschultz & Veeman, 1998; Unterschultz, Quagrainie & Vincent, 1997). In addition, region of origin has been examined in many studies (Grunert, 1997; Mennecke et al., 2007; Mesías et al., 2005; Quagrainie et al., 1998; Unterschultz et al., 1997) and with a few exceptions (Grunert, 1997), the results indicate that region of origin is an important factor influencing consumer attitudes about meat products (Mennecke et al., 2007). In all of the studies where region of origin was found to be important, the results show that consumers or meat buyers prefer beef that come from local producers or from the country or region in which the consumer resides (Mennecke et al., 2007; Mesías et al., 2005; Quagrainie et al., 1998; Unterschultz et al., 1997). Results from this study also showed that the highest utility for the consumers resulted from beef produced locally. The lower relative importance of geographical origin in this study compared with other studies carried out in Spain (Mesías et al., 2005; Realini et al., 2013; Sánchez et al., 2012), may indicate that when choices include foreign origin the importance of this extrinsic cue is higher than when only national options are evaluated.

Previous studies suggested that colour of lean muscle tissue, visible fat content, flavor, and tenderness are important cues for consumers' beef purchase decisions (Huffman, Miller, Hoover, Wu, Brittin & Ramsey, 1996; Lusk, Fox, Schroeder,

Mintert & Koohmaraie, 2001). However, from these attributes only colour and fat content of beef can be used as cues to infer meat quality at the point of purchase. Many authors indicated that the colour of fresh red meat is of the utmost importance in meat marketing since it is the first quality attribute seen by the consumer who uses it as an indication of freshness and wholesomeness (Faustman et al., 1990; Glitsch, 2000). At the point of sale, colour and colour stability are the most important attributes of meat quality and various commercial approaches have been used to meet consumer expectation as consumers will discriminate negatively against meat that does not appear to match expectations or that is discoloured (Hood & Mead, 1993). In fact, (Taylor, 1996) indicated that the colour of fresh meat is not well correlated with the eating quality, however, the consumer still demands beef to be a bright cherry-red colour. (Bello Acebrón et al., 2000; Grunert, 1997) found that light meat colour is preferred over dark meat, while (Steenkamp et al., 1996) found a positive evaluation when redness increases in agreement with the results from the present study where respondents preferred bright red over pale red beef except for consumers from Zaragoza who thought that the colour of fresh beef was not important for their purchasing decisions.

European consumers are interested in how food is produced and (Verbeke et al., 2010) showed that healthy beef was associated with the production system depending on how animals were fed and kept in the eyes of the consumers. (Napolitano, Braghieri, Piasentier, Favotto, Naspetti & Zanoli, 2010) indicated that the information about production systems can be a determinant of beef preference, thus providing a potential tool for meat differentiation. According to (Grunert, Verbeke,

Kügler, Saeed & Scholderer, 2011) new product development in the meat sector involves the whole value chain and animal production is getting more differentiated representing the first opportunity to modify the presence of bioactive components (Olmedilla-Alonso et al., 2013). Meat composition varies with respect to numerous factors and animal diet is the factor which can most easily be manipulated and which has one of the most profound effects on its composition (Troy et al., 2010). Thus, animal feeding strategies involving plant (vegetable oils, n−3 PUFA-rich plants, forages) and marine sources (fish or algae) have been successfully used to significantly increase polyunsaturated fatty acids (Raes, De Smet & Demeyer, 2004; Scollan, Hocquette, Nuernberg, Dannenberger, Richardson & Moloney, 2006; Wood, Enser, Richardson & Whittington, 2008), and dietary supplementation has been used to enrich beef with CLA (Gillis, Duckett, Sackmann, Realini, Keisler & Pringle, 2004; Raes et al., 2004; Schmid, Collomb, Sieber & Bee, 2006).

Animal feeding has been identified as an important factor in consumers' beef purchasing decisions (Olaizola Tolosana et al., 2005; Realini et al., 2013), however, in the current study it was not considered important except for consumers from Zaragoza who indicated that beef enriched with CLA was the least preferred type of beef. Most consumers are familiar with *n-3* fatty acids, however, CLA is not commonly known by consumers and no information about *n-3* or CLA was provided in this study. Thus, the lowest preference by respondents from Zaragoza for CLA enriched beef over other types of beef (conventional and enriched with *n-3* or *n-3* plus CLA), may be linked to the lack of consumers' association of CLA and the positive association of *n-3* fatty acids with human health. This study shows that

consumers are willing to pay a premium over 14€/kg of 1.21, 1.52 and 2.04 € on average for CLA, *n-3* and *n-3* plus CLA enriched beef loin. There was no difference in the WTP for *n-3* enriched beef among cities, but willingness to pay was higher for consumers from Barcelona and Pamplona than Zaragoza for CLA enriched beef and for consumers from Barcelona than Zaragoza and Pamplona for *n-3* plus CLA enriched beef. In addition, consumers from Zaragoza and Pamplona showed lower probability to purchase enriched meat compared with consumers from Barcelona. Thus, marketing efforts for enriched beef would be more successful in Barcelona, followed by Pamplona and lastly Zaragoza. Consumers from Barcelona did not find sensory differences among the different types of enriched beef. However, beef enrichment with *n-3* or CLA seems to have sensory advantages, but beef enrichment with both *n-3* and CLA offers no sensory advantages over conventional beef for most consumers. To the best of our knowledge, there are no published data regarding the effect of the combined enrichment with CLA and *n-3* fatty acids on consumer sensory acceptability of beef.

4.2 Cluster analysis

Three respondent segments were identified based on the pattern of individual utilities estimated by choice analysis for all evaluated consumers (n=322, Table 3). A number of differences among consumer clusters have been identified, and some of these differences are associated with socio-demographic characteristics (Table 5) of the respondents and their attitudes and behavior towards beef, health concerns, willingness to pay and purchase probability of enriched meat (Table 6).

Cluster 1 (44% of sample) consist of consumers that attach more importance to the fat content of beef followed by origin with preference for beef with low fat level and locally produced. This consumer segment can be labeled as 'health-conscious consumers' and is equally distributed among all the cities in the study. This cluster is characterized by having a higher percentage of women and retired and housewife respondents and a higher average age. Respondents also indicated a lower frequency of beef purchase at the supermarket and a higher food expenditure. In addition, consumers in this cluster have higher cholesterol level, check their health more regularly and have blood analysis done more recently. Consumers in this cluster are more used to purchase enriched food, are more willing to pay for enriched beef and are more likely to purchase enriched meat. Consistent with the results from this study, (Moskowitz et al., 2004) indicated that persons who are strong believers in the health benefits of foods tend to be female and in the 35-64 age group. (Peng et al., 2006) also indicated that the consumer target segment for most CLA-enriched milk products can be characterized as being health-conscious and middle-aged consumers. According to (Krystallis, Maglaras & Mamalis, 2008), the early-middle aged consumers emphasize more on knowing the origin of the functional product and disease prevention attributes such as lower cholesterol, reduced cardiovascular disease risk and low saturated fatty acid content, while young adults are more interested in convenience and low price. Other authors have also shown that women are more cautious and prefer the locally produced beef (Alfnes, 2004; Realini et al., 2013) and lamb (Font i Furnols et al., 2011) compared with men. (Mesías et al., 2005) identified a cluster of beef consumers that placed great importance to origin and little importance to price and quality. This cluster had the greatest percentage of

frequent beef consumers, characterized by a high proportion of housewives, by an older age of its members, and by having the greatest percentage of individuals with no formal studies in agreement with results from the current study. (Papanagiotou et al., 2013) also indicated that origin's importance increases with the increase of age while the reverse appears to be true for education. Studies conducted with CLA enriched dairy products support the results obtained in this study with *n-3* and/or CLA enriched beef, showing that consumers who are more health conscious are the ones who declare a greater WTP for enriched foods (Di Pasquale, Adinolfi & Capitanio, 2011; Maynard & Franklin, 2003; Peng et al., 2006).

The second segment (31% of sample) can be labeled 'price-oriented consumers' as respondents are most concern about meat price and fat content with preference for lowest meat price and lean beef. This segment has a high proportion of respondents from Zaragoza and Pamplona and is characterized by consumers with the lowest average age and higher education level associated with a higher percentage of respondents as students. This cluster has lower beef and lamb consumption, higher frequency of meat purchase at supermarket, and lower food expenditure than respondents in cluster 1. Moreover, these respondents are less concern about health, are less willing to pay for enriched meat than consumers in cluster 1 and are less likely to buy enriched meat. Previous research has also shown that young consumers are more influenced by price in their purchase intentions of meat (Font i Furnols et al., 2011; Krystallis et al., 2008; Realini et al., 2013). (Papanagiotou et al., 2013) indicated that the usage of the extrinsic cue 'price' for the formation of quality judgments is an indication of uncertainty and perceived difficulty in quality

evaluation (Bredahl, 2004; Papanagiotou et al., 2013). Younger consumers with lower frequency of beef consumption who buy meat more often at supermarkets may be less likely to judge meat quality by relying on intrinsic cues. (Bernués, Ripoll & Panea, 2012) indicated that intrinsic quality attributes such as colour and appearance of freshness are important for more traditional consumers, who are more likely than average to have a lower educational level, and to be older.

Finally, cluster 3 (25% of sample) consists of consumers that consider fat content as the most important quality cue followed by meat price. This smaller but significant segment of consumers is the only group with preference for higher visible fat content and higher meat price which are both attributes associated with a higher eating quality. Thus, this cluster can be labeled as 'quality-oriented consumers'. This cluster has a high proportion of respondents from Barcelona and a higher percentage of men than the other segments. Members in this cluster showed intermediate characteristics in terms of average age, health concerns and probability to purchase enriched meat relative to clusters 1 and 2. They are also less willing to pay for enriched beef than consumers in cluster 1. (Papanagiotou et al., 2013) recently reported that males prefer to buy pork with some marbling resulting from their interest in the sensory attributes of meat and the satisfaction they derive from its consumption. Origin was not an important attribute in the beef purchasing decisions of respondents in cluster 3, which is in agreement with other studies that showed that men are less influenced by this extrinsic attribute than women (Alfnes, 2004; Font i Furnols et al., 2011; Realini et al., 2013).

Although animal diet was the third most important attribute for all segments, its relative importance did not differ from meat colour in any segment or from meat price in cluster 1. The 'health conscious' consumers were the only ones that preferred the *n-3* enriched beef, while consumers in clusters 2 and 3 preferred the conventional over any type of enriched beef. In addition, consumers in cluster 1 assigned higher sensory scores to *n-3* enriched beef than conventional or *n-3* plus CLA enriched beef indicating that 'health conscious' consumers are a potential target for enriched beef. It is also evident from cluster analysis that the individual beef enrichment with *n-3* or CLA offers sensory advantages while the combined enrichment with *n-3* plus CLA offers no sensory advantages over conventional beef. A similar relative importance was assigned by consumers in each cluster to the 'colour' intrinsic quality attribute of beef, and bright red meat was preferred over pale red colour by all evaluated consumers. (Steenkamp et al., 1996) also reported that the evaluation of a positive appearance of meat increases as redness increases. Finally, other socio-demographic variables such as income level did not help to discriminate between the three identified segments. (Di Pasquale et al., 2011) recently indicated that income level was not associated with any consumer cluster and a greater WTP for functional foods is strongly linked to the type of product carrier but not greatly to income.

5. Conclusions

The most important cue driving the majority of consumers' beef purchase decisions was the visible fat content followed by price, then origin and colour, and finally animal diet which was not important. Most consumers preferred beef with a slight

content of visible fat, but consumer dissatisfaction may occur when beef is consumed. Thus, identifying an optimum fat level to achieve a visually acceptable marbling that does not compromise sensory quality may contribute to reduce the gap between expected and experienced quality of beef. Medium-low meat price was preferred over the lowest priced beef, and consumers were willing to pay a premium of 1.21, 1.52 and 2.04€ over 14€/kg for CLA, *n-3* and *n-3* plus CLA enriched beef, respectively. Locally produced beef was preferred by all consumers indicating that protected geographical indication and brands promoting local beef would have marketing potential. Bright red colour was also preferred by all consumers over pale red beef. Cluster analysis revealed three consumer profiles: 'health conscious consumers' with preference for lean beef and represented by older consumers, women, retired, housewives, greater food expenditure and more concerned with health; 'price-oriented consumers' with preference for lowest priced beef represented by younger consumers, students, higher education, lower beef intake and purchase at supermarket; and 'quality-oriented consumers' with preference for beef with moderate amount of visible fat represented by consumers from Barcelona and men. The individual beef enrichment with *n-3* or CLA improved sensory acceptability scores, while the combined enrichment with *n-3* plus CLA offered no sensory advantages over conventional beef.

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Table 1. Demographic characterization of consumers by city and for all cities.

	Barcelona	***Zaragoza***	***Pamplona***	***Total***
N	100	113	109	322
Gender (%)				
Men	54.0	50.4	45.9	50.0
Women	46.0	49.6	54.1	50.0
Age (%)				
18-25	12.0	31.0	36.7	27.1
26-40	25.0	22.1	16.5	21.1
41-60	45.0	28.3	29.4	33.9
61-75	18.0	18.6	17.4	18.0

Table 2. Consumers' attitudes and behaviour included in the survey.

Variables	***Description***
Consumption Behaviour	
How many times per week do you consume the following types of meat? Beef, Lamb, Pork, Chicken	Indicate times per week
Where do you purchase fresh beef? Butcher, Supermarket, Traditional market	Indicate one or more options
How much money do you spend in food at home each month? < 200 €, 200-400 €, >400 €	Indicate one option
Consumers' health concerns	
Do you have high cholesterol level?	Indicate YES or NO
When was the last time that you had a blood analysis?	Indicate moths since the last analysis
I check my health status regularly	Indicate the degree of agreement or disagreement with this statement using a 9 point scale from 1: I totally agree to 9: I totally disagree
I am used to purchase enriched food products with vitamins, calcium, omega-3, etc (For example: milk and dairy products, eggs, cookies, etc)	Indicate the degree of agreement or disagreement with this statement using a 9 point scale from 1: I totally agree to 9: I totally disagree
Consumers' willingness to pay	
Considering that the average price for a beef loin steak in Spain is 14 €/kg, how much more would you be willing to pay for the following different types of enriched beef steaks? Enriched with CLA, Enriched with omega-3, Enriched with omega-3 plus CLA	Indicate in €/kg above 14 €/kg
Probability to purchase enriched meat	
Which would be the probability that you would buy the following types of enriched meat if they were available commercially where you normally buy meat? Beef, Lamb, Pork, Chicken	Indicate using a 5 point scale where 1: extremely likely, 2: likely, 3: unlikely, 4: extremely unlikely, and 5: I would not buy

Table 3. Relative importance of each factor (origin of production, animal diet, fat content, beef colour, beef price) and utilities for each level of the factors for consumers from Barcelona, Zaragoza and Pamplona.

All Consumers	***Barcelona***	***Zaragoza***	***Pamplona***
N	**100**	**113**	**109**
	Relative Importance (%) and Confidence Interval at 95%		
Origin (3)	**5.42**^{by} (-2.5,13.3)	**21.45**^{ay} (9.6, 33.3)	**21.67**^{ax} (14.0, 29.4)
Diet (4)	**9.12**^{by} (-6.3,24.5)	**17.63**^{ay} (0.9, 34.3)	**1.22**^{by} (-8.7, 11.2)
Fat content (1)	**30.02**^{ax} (14.0, 46.1)	**34.01**^{ax} (10.5, 57.5)	**27.96**^{ax} (16.1, 39.8)
Colour (2)	**24.49**^{ax} (14.4,34.6)	**2.04**^{bz} (-16.4, 20.5)	**22.40**^{ax} (11.9, 32.9)
Price (5)	**30.95**^{ax} (18.9, 43.0)	**24.87**^{bx} (5.4, 44.3)	**26.76**^{bx} (16.9, 36.6)
	Random Parameters Logit Model (RPL)		
	Utilities		
Origin			
Not locally produced	-0.09	-0.27***	-0.39***
Locally produced	0.09	0.27***	0.39***
Diet			
Conventional	-0.17	0.22	-0.01
Enriched with omega-3	-0.05	0.06	-0.02
Enriched with CLA	0.13	-0.21*	0.02
Enriched with omega-3 and CLA	0.09	-0.07	0.02
Fat Content			
Moderate visible fat	-0.50***	-0.42***	-0.50***
Slight visible fat	0.50***	0.42***	0.37***
Colour			
Pale red	-0.40***	-0.03	-0.40***
Bright red	0.40***	0.03	0.40***
***Price* (€ per tray, 0.3 kg)**			
6.6 (high)	-0.52***	-0.41***	-0.55***
5.7 (medium-high)	0.29**	0.08	0.12
4.8 (medium-low)	0.50***	0.11	0.42***
3.9 (low)	-0.27*	0.21	0.01
No option	0.06	0.94***	0.72***
LL(0)	-1109.0	-1253.2	-1208.8
LL(θ)	-857.5	-1007.9	-998.5
LLR	502.9 (0.000)	490.5 (0.000)	420.6 (0.000)
McFadden Pseudo R^2	.226	0.195	0.174
Observations in Barcelona =3200=(100 consumers ×4 alternatives ×8 choice sets)			
Observations in Zaragoza = 3616= (113 consumers ×4 alternatives ×8 choice sets)			
Observations in Navarra = 3488= (109 consumers ×4 alternatives ×8 choice sets)			

Cities with different superscript letters in rows (^{a,b}) and factors with different superscript letters in columns (^{x,y,z}) differ (P < 0.05).

Table 4. Relative importance of each factor (origin of production, animal diet, fat content, beef colour, beef price) and utilities for each level of the factors for all consumers and for each cluster.

All Consumers	***Total Sample***	***Cluster 1***	***Cluster 2***	***Cluster 3***
N	**322**	**141**	**99**	**82**
	Relative Importance (%) and Confidence Interval at 95%			
Origin	**17.28**^{y} (11.8, 22.7)	**17.64**^{ax} (12.3, 23.0)	**6.19**^{by} (1.3, 11.0)	**1.71**^{bz} (-3.7, 7.2)
Diet	**3.52**^{z} (-3.8, 10.9)	**9.64**^{ay} (1.5, 17.8)	**13.25**^{ax} (6.1, 20.4)	**16.00**^{ay} (9.4, 22.6)
Fat content	**35.70**^{w} (26.1, 45.3)	**54.53**^{aw} (45.9, 63.1)	**31.30**^{bw} (23.9, 38.7)	**47.08**^{aw} (39.8, 54.4)
Colour	**19.02**^{y} (11.9, 26.2)	**8.28**^{ay} (2.1, 14.5)	**12.31**^{ax} (6.6, 18.0)	**13.74**^{ay} (8.9, 18.5)
Price	**24.48**^{x} (17.5, 31.5)	**9.91**^{cy} (1.0, 18.8)	**36.96**^{aw} (28.8, 45.1)	**21.48**^{bx} (15.4, 27.5)
	RPL Model	***LC Model***		
	Utilities	***Utilities***		
Origin				
Not locally produced	-0.24***	-0.23^{***b}	-0.16^{***b}	-0.04^{a}
Locally produced	0.24***	0.23^{***b}	0.16^{***b}	0.04^{a}
Diet				
Conventional	0.01	-0.09^{b}	0.42^{***a}	0.29^{**a}
Enriched with omega-3	0.03	0.16^{**a}	-0.05^{b}	0.11^{b}
Enriched with CLA	-0.02	-0.09^{b}	-0.12^{b}	-0.50^{***a}
Enriched with omega-3 and CLA	-0.02	0.02^{b}	-0.25^{**a}	0.11^{b}
Fat Content				
Moderate visible fat	-0.53***	-0.71^{***b}	-0.79^{**b}	1.16^{***a}
Slight visible fat	0.53***	0.71^{***b}	0.79^{***b}	-1.16^{***a}
Colour				
Pale red	-0.25***	-0.11^{**b}	-0.31^{***a}	-0.34^{***a}
Bright red	0.25***	0.11^{**b}	0.31^{***a}	0.34^{***a}
***Price* (€ per tray, 0.3 kg)**				
6.6 (high)	-0.52***	0.01^{a}	-1.13^{***c}	-0.53^{***b}
5.7 (medium-high)	0.09	0.11^{c}	-0.23^{*b}	0.53^{***a}
4.8 (medium-low)	0.34***	0.02^{c}	0.63^{***a}	0.23^{**b}
3.9 (low)	0.08	-0.15^{**b}	0.73^{***a}	-0.22^{*b}
No option	0.53***	-1.54***	1.37***	-0.424***
N =10.304=(322 consumers ×4 alternatives ×8 choice sets)		**Estimated Latent Class Probabilities**		
		0.43***	0.31***	0.25***
LL(0) = -3571.09, LL(θ)= -2939.3		LL(0) = -3,571.09, LL(θ)= -2,979.38		
LLR=1263.6 (0.000), Pseudo R^2= 0.177		LLR= 1,183.4 (0.000), Pseudo R^2= 0.165		

Cities with different superscript letters in rows (^{a,b}) and factors with different superscript letters in columns (^{w,x,y,z}) differ (P < 0.05).

LL(0) Restricted Log likelihood at constant, LL(θ) Log likelihood of the model, LLR: Log Likelihood ratio test. RPL: Random Parameters Logit Model, LC: Latent Class Model. Significance levels: *** p<0.01; **p<0.05; * p< 0.10.

Table 5. Least squares means and standard error (SE) of overall acceptability of beef from animals fed different diets (CON: conventional, OME3: omega-3, CLA: conjugated linoleic acid, OME3CLA: omega-3 plus conjugated linoleic acid) by consumers from Barcelona, Zaragoza and Pamplona individually and across cities.

	Diets				
	CON	OME3	CLA	OME3CLA	SE
Barcelona ***(n=100)***	6.02[a]	6.41[a]	6.05[a]	6.01[a]	0.178
Zaragoza ***(n=113)***	5.77[b]	6.19[a]	6.20[a]	5.73[b]	0.154
Pamplona ***(n=109)***	5.41[b]	5.94[a]	5.87[a]	5.48[b]	0.158
All cities ***(n=322)***	5.73[b]	6.17[a]	6.04[a]	5.73[b]	0.094
Cluster 1 (**n=141**)	5.99[b]	6.51[a]	6.26[ab]	5.86[b]	0.146
Cluster 2 (**n=99**)	5.61[ab]	5.93[a]	5.88[ab]	5.55[b]	0.169
Cluster 3 (**n=82**)	5.43[a]	5.89[a]	5.87[a]	5.72[a]	0.180

[a,b] Means within rows with different superscript letters differ ($P < 0.05$).

Table 6. Socio-demographic characterisation of consumers and their clusters.

	All consumers	***Cluster 1***	***Cluster 2***	***Cluster 3***
N	322	141	99	82
Gender (%)				
Men	50.0	43.3^{c}	52.5^{b}	58.3^{a}
Women	50.0	56.7^{a}	47.5^{b}	41.5^{c}
Age (%)				
18-29	27.1	18.4^{c}	35.4^{a}	31.7^{b}
30-40	21.1	17.7^{b}	32.3^{a}	13.4^{c}
41-60	33.9	36.9^{a}	28.3^{c}	35.4^{b}
> 60	18.0	27.0^{a}	4.0^{c}	19.5^{b}
Age (years)	43.4	47.8^{a}	36.6^{c}	44.0^{b}
Education Level (%)				
Primary studies or less	12.7	19.8^{a}	3.0^{b}	12.2^{a}
Secondary studies	32.0	32.6^{a}	27.3^{a}	36.6^{a}
University studies	55.3	47.5^{b}	69.7^{a}	51.2^{b}
Income level (%)				
Less than the mean	37.3	37.6^{a}	37.4^{a}	36.6^{a}
Equal to the mean	49.7	46.8^{a}	54.5^{a}	48.8^{a}
Higher than the mean	13.0	15.6^{a}	8.1^{a}	14.6^{a}
Occupation (%)				
Student	21.1	12.1^{b}	30.3^{a}	25.6^{a}
Employee	41.3	41.8^{a}	43.4^{a}	37.8^{a}
Business Owner	4.3	2.8^{a}	7.1^{a}	3.7^{a}
Retired	13.0	17.7^{a}	4.0^{b}	15.9^{a}
Housewife	7.5	13.5^{a}	1.0^{b}	4.9^{b}
Unemployed	12.7	12.1^{a}	14.1^{a}	12.2^{a}
Cities (%)				
Barcelona	31.1	34.8^{b}	18.2^{c}	40.2^{a}
Zaragoza	35.1	32.6^{b}	37.4^{a}	31.7^{b}
Pamplona	33.9	32.6^{b}	44.4^{a}	28.0^{c}

^{a,b,c} Means within rows by cluster with different superscript letters differ ($P < 0.05$).

Table 7. Attitudes and behaviour of consumers and their clusters.

	All consumers	*Cluster 1*	*Cluster 2*	*Cluster 3*
N	322	141	99	82
Consumption behaviour				
Meat consumption (times/week)				
Beef	1.46	1.57[a]	1.30 [b]	1.47[a]
Lamb	0.58	0.62 [a]	0.50 [b]	0.62 [a]
Pork	1.41	1.31[a]	1.50[a]	1.47 [a]
Chicken	1.90	1.96 [a]	1.87 [a]	1.84 [a]
Place of meat purchase (%, options are non-exclusive)				
Butcher	71.4	73.0[a]	66.7[a]	74.4[a]
Supermarket	59.0	53.9[b]	65.7[a]	59.8[a]
Traditional market	25.2	29.1[a]	19.2[b]	25.6[a]
Food expenditure in €/month (%)				
< 200	34.5	28.4[b]	38.4[a]	40.2[a]
200-400	41.9	41.1[a]	45.5[a]	39.0[a]
> 400	23.6	30.5[a]	16.2[b]	20.7[b]
Consumers' health concerns				
I have high cholesterol level (%)	15.5	21.3[a]	9.1[c]	13.4[b]
I check my health periodically (1-9 Likert scale)[A]	6.19	5.68[a]	3.84[c]	4.93[b]
I am used to purchase enriched food (1-9 Likert scale)[A]	4.92	6.53[a]	5.72[c]	6.17[b]
Months since the last blood analysis	7.12	6.49[c]	7.97[a]	7.18[b]
Consumers' willingness to pay (WTP)				
WTP for different types of enriched beef meat (€ above 14 €/kg)				
CLA	1.20	1.49[a]	1.01[a]	0.94[a]
Omega-3	1.52	1.98[a]	1.19[b]	1.12[b]
Omega-3 and CLA	2.02	2.73[a]	1.38[b]	1.59[b]
Probability to purchase enriched meat with omega-3 and/or CLA (1 to 5 Likert scale)[B]				
Beef	2.66	2.13[c]	3.30[a]	2.78[b]
Lamb	3.05	2.57[c]	3.66[a]	3.16[b]
Pork	3.01	2.59[b]	3.53[a]	3.10[a]
Chicken	2.88	2.38[c]	3.51[a]	2.98[b]

[a,b,c] Means within rows by cluster with different superscript letters differ (P < 0.05).
[A] From 1: I totally disagree to 9: I totally agree. [B] From 1: Very likely to 5: I would not buy.

Figure 1. Example of a choice set.

6. Aptitud tecnológica

Shelf life of ground beef enriched with omega-3 and/or CLA and use of grape seed extract to inhibit lipid oxidation

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Short version of title: (ground beef with n-3, CLA and GSE)

Section: Sensory and Food Quality

ABSTRACT

The shelf life and oxidative stability of refrigerated raw ground beef enriched with omega-3 and/or conjugated linoleic acid (CLA) was studied. Grape seed extract (GSE) was used to inhibit the lipid oxidation in the ground beef. Eight treatments of ground beef were established according to the enrichment of beef (control, enriched with omega-3, with CLA, or with omega-3 plus CLA) and the use of GSE (0 and 250 mg GSE/kg product). Fresh beef was ground and mixed with GSE and salt. Treatments of beef were stored at 2 ± 1 ºC in aerobic packaging for 0, 1, 3 and 6 days under retail display conditions. Oxidation stability (TBARS), pH, instrumental color, metmyoglobin formation and sensory attributes (color and odor) were measured. Omega-3 enriched beef increased the oxidation level at day 6 as determined by TBARS (P<0.05) but the instrumental color was not affected. The enrichment of CLA improved the coordinates of color (P<0.05) until day 3 and decreased the oxidation at day 6 (P<0.05). There were not differences for color and odor values among type of beef during display, except at day 3, when CLA treatments were the best valuated. Addition of GSE decreased the oxidation level (P<0.001) and did not affect the instrumental color neither the sensory parameters.

Keywords: ground beef, omega-3, CLA, grape seed extract, shelf life

Practical Application: The beef enriched with omega-3 and/or CLA presents appropriate technological aptitude. The enrichment of beef with polyunsaturated fatty acids promotes the lipid oxidation, which could be solved with grape seed extract. Thus, the use of this beef enriched would lead new meat products, more competitive in meat sector and closer to current nutritional recommendations.

Introduction

Consumers are more health-conscious, driving a trend towards nutritious foods with additional health promoting functions. In general, meat and meat products are essential in the diet of developed countries (Fernández-Ginés and others 2005) and its health attributes can be improved by increasing the omega-3 (n-3) and CLA fatty acids. These fatty acids influence on meat shelf life due to the propensity of unsaturated fatty acids to oxidize, leading to the development of rancidity and off-odor as display times increases (St. Angelo and others 1990). Moreover, technological operations of meat processing can alter its quality. For instance, ground beef is more bound to color deterioration and oxidation than are its whole muscle counterparts (Honikel 2004). Because color and oxidation stability are very important to retail shelf life, the use of antioxidants is necessary.

The use and applications of natural antioxidants is increasing because studies indicate possible adverse health effects from the use of synthetic antioxidants. Polyphenols are a type of natural antioxidants that, in addition to its antioxidant properties in raw meats (Chen and others 1999), have specific biological activities that provide beneficial and healthy effects for the human body (Gharras 2009). Grape seed extracts (GSE) are a rich source of polyphenols compounds especially phenolic acids, flavan-3-ols such as catechins and their isomers and proanthocyanidins. The GSE has shown antioxidant activity in beef (Ahn and others 2002; Bañón and others 2007; Rojas and Brewer 2007; Rojas and Brewer 2008; Schevey and others 2013). The antioxidant activity of GSE is dependent on its concentration from 0.02% to 0.1% (Ahn and others 2002). Gómez and others (2011) studied several concentrations of GSE in raw beef patties and concluded that 500 mg GSE/kg meat

was enough to prevent rancidity of raw beef patties packaged in air and stored for 10 days under retail display conditions.

Therefore, the use of GSE can help to improve the shelf life of ground beef enriched with n-3 and CLA without affecting oxidative stability or cause adverse effects on sensory characteristics, thus offering a more competitive product on the market. The aim of the present study was to examine the shelf life and oxidative stability in refrigerated raw ground beef enriched with omega-3 and/or CLA. Grape seed extract was also used as natural antioxidant to inhibit the lipid oxidation in the ground beef enriched with omega-3 and/or CLA.

2. Material and methods

2.1. Materials

2.1.1. Beef

Beef loin cuts were obtained at 24 h *postmortem* from the right carcass side of 48 Holstein entire males (10.7 months old) fed with one of four dietary treatments. All animal diets had similar composition but differed in the content of whole linseed and conjugated linoleic acid (CLA): Control (C, conventional commercial ration, 0% linseed and 0% CLA), omega-3 (OME3, conventional ration enriched with omega-3 fatty acids through the addition of 10% linseed), CLA (CLA, conventional ration enriched with CLA through the addition of 2% CLA), and omega-3+CLA (OME3+CLA, conventional ration enriched with omega-3 and CLA fatty acids through the addition of 10% linseed plus 2% CLA). Animal productive performance and carcass characteristics of these animals were reported by Albertí and others (2013). Animals were slaughtered with an average live weight of 458.4 ± 16.6 kg at

an EU-licensed commercial abattoir following standard procedures. Vacuum packaged loin cuts were transported to the Public University of Navarre meat laboratory and they were stored at –18 ºC until required for the experiment. The proximate composition and the fatty acid content of loin cuts are shown in Table 1.

2.1.2. Extract

A commercial grape seed extract (GSE) with a polyphenol content of 95% was used. GSE was provided by Exxentia (Madrid, Spain) and it was a water soluble homogeneous brown powder. The use of GSE (GSE-0 and GSE-250, 0 and 250 mg GSE/kg meat respectively) on ground beef was studied. The selection of the dose used (250 mg GSE/kg meat) was based on a previous study (Gómez and others 2011).

2.2. Preparation of ground beef

Eight treatments (Table 2) of ground beef were established according to beef enriched PUFA (C, OME3, CLA and OME3+CLA) and the use of GSE (GSE-0 and GSE-250): C–GSE-0, OME3–GSE-0, CLA–GSE-0, OME3+CLA–GSE-0, C–GSE-250, OME3–GSE-250, CLA–GSE-250 and OME3+CLA–GSE-250.

The frozen beef loin cuts were allowed to thaw 24 h before being minced. The twelve beef loin cuts from each one of four dietary treatments (C, OME3, CLA, OME3+CLA) were minced together through a Cato mincer (TALSABELL S.A., Sabadell, Spain). The minced beef (C, OME3, CLA, OME3+CLA), salt (2%) and GSE (0, 250 mg GSE/kg product) were then blended together by a Sammic mixer (Sammic S.L., Azkoitia, Spain) for 60 seconds. The mix was then weighed into portions of 100 g and formed in to patties between grease proof papers using a patty

press, to give average dimensions of 10 cm diameter and 1.5 cm thickness. The meat temperature during processing did not exceed 7 ºC. The patties were placed in transparent plastic trays covered with transparent polyvinyl chloride film (PVC) and stored at 2 ± 1 ºC for 6 days in a display cabinet illuminated (10 hours/day) with white fluorescent light, simulating retail display conditions. On each evaluation day (0, 1, 3 and 6), samples were prepared for pH, color, TBARS, and sensory analyses.

2.3. pH values

The pH of the treatments at 0 and 6 days of the display were measured (AOAC, 2003) in quadruplicate for each sample. The pH was measured by homogenization in water using a Crison GLP22 pHmeter (Crison Instruments S.A., Barcelona, Spain) equipped with a 6 mm (diameter) penetration probe.

2.4. Color

Color was measured using a Minolta CM-2002 spectrophotometer (Konica Minolta Business Technologies Inc., Tokyo, Japan) making five measurements per sample. Color parameters were evaluated directly on the patties surface during display (0, 1, 3 and 6 days) using the CIE L*a*b* system, illuminant D65 and 10 º as the standard observing point. Results were expressed as CIELab values: Lightness (L*), Redness (a*), Yellowness (b*), Chroma (C*) and Hue angle (H*); $C = (a^{*2} + b^{*2})^{0.5}$; H = arctg b* / a*. The accumulation of metmyoglobin (MMb) on the meat surface was followed by calculating the $K/S_{572} \div K/S_{525}$ ratio using the reflectance values, according to Hunt and others (1991).

2.5. Thiobarbituric acid-reactive substances

Thiobarbituric acid reactive substances (TBARS) values were determined, at the 0 and 6 days of the display, in duplicate for each sample, using the method described by Tarladgis and others (1960). The absorbance was measured at 538 nm in a spectrophotometer (UV-2101PC, Shimadzu, Kyoto, Japan). The TBARS value, expressed as the mg malonaldehyde/kg meat, was obtained using a conversion factor based on a standard curve using 1,1,3,3-tetraethoxypropane (TEP).

2.6. Raw ground beef sensory analysis

A sensory analysis was made on the raw ground beef at the 0, 1, 3 and 6 days of the display. The methodology carried out to assess beef odor and color degradation was adapted from the one described by Insausti and others (2001). A semi-trained sensory panel of 15 panelists was recruited from Public University of Navarre. Firstly, odor evaluations were performed under soft red light (≈ 100 lux), and then color evaluations were evaluated under white light (≈ 450 lux). Panelists rated odor and color using a 15 cm line anchored at each end with the terms: on the left side “non detectable off-odor”, “bright fresh red meat” and on the right side “extreme off-odor”, “brown, greenish, discolored meat”, respectively. The acceptability limit was anchored in the middle of the line (7.5 cm from each end). The results were quantified by measuring the distance in cm of the Panelists mark from the left side.

2.7. Cooked ground beef sensory analysis

Samples were taken from each of the treatments at 2 days of the display. Samples were cooked in a double hot-plate grill pre-heated to 200 ºC until final internal temperature reached 71 ºC that was determined using individual thermocouples inserted into the geometric centre of the meat. Samples were coded and kept warm in a heater for between 5 and 15 min until sensory analyses. The methodology carried out to assess cooked beef odor and color degradation was the one described previously for raw ground beef. Panelists rated odor and color using a 15 cm line anchored at each end with the terms: on the left side “non detectable off-odor”, “typical aspect cooked meat” and on the right side “extreme off-odor”, “discolored cooked meat”, respectively.

2.8. Statistics

Two master batches of each treatment were manufactured producing two replications of the entire experiment. Data were analyzed using a general linear model (GLM) procedure (IBM-SPSS version 21 for Windows). The effect of the replicate was not significant between the two batches from each the treatments. For pH, TBARS, instrumental color and sensory data of raw ground beef, the statistical model included the fixed effects of beef (B), grape seed extract (GSE) and storage time (T), as well as their interactions and residual error. For sensory data of cooked ground beef, the statistical model included the fixed effects of B and GSE, as well as their interaction and residual error. Differences among means were analyzed by the Tukey procedure. Pearson’s correlation coefficients between variables were calculated. For all tests, the level of significance was set at $P<0.05$. Multivariate analysis, namely, factor analysis, was used to examine the relationships among all

the variables studied. Factors were extracted using the principal component analysis (PCA) method. The Varimax rotation method was applied to the factors to facilitate interpretation and to maximise the explained variance.

3. Results and Discussion

3.1. pH

Table 3 shows the pH values of the treatments, whose triple interaction BxGSExT was not significant ($P>0.05$). The interaction BxGSE and the effect of B were statistically significant ($P<0.05$) at 0 day, whereas only the beef had significant effect ($P<0.05$) on pH values at 6 day. The omega-3 enriched beef had lower pH values than those of control beef (5.25 *vs* 5.32; $P<0.05$). However, in previous studies there were no differences on pH of beef from bulls fed linseed (Juárez and others 2012). The pH values were not affected by time in the raw ground beef, except the OME3+CLA treatment that had lower pH values at 6 day. GSE supplementation had no significant effect on pH during the display; likewise, Bañón and others (2007) and Rojas and Brewer (2007) also found no differences when GSE was added in ground beef.

3.2. TBARS

Table 4 shows the level of lipid oxidation of the treatments. There was a significant interaction BxGSExT ($P<0.001$) for TBARS of ground beef during display. Beef and GSE factors as well as the interaction of BxGSE had no significant effects ($P>0.05$) on lipid oxidation at 0 day. Nevertheless, interaction of BxGSE and

beef and GSE factors were statistically significant (P<0.001) at 6 day. TBARS values of treatments without GSE increased gradually from 0.57 mg MDA/kg to around 3.24 mg MDA/kg (P<0.001) during display, whereas treatments with GSE had constant TBARS values (around 0.53 mg MDA/kg; P>0.05) and lower than the value of 2 mg MDA/kg, which is the upper limit of rancidity for the acceptability of beef consumer (Campo and others 2006).

The treatments without GSE that suffered the highest oxidation level were which used beef enriched with omega-3 (4.51 mg MDA/kg) because polyunsaturated fatty acids are more susceptible to the lipid oxidation and decrease the lipid stability for refrigerated storage. Previous studies reported that steaks with higher omega-3 fatty acid (FA) content (from flaxseed diets) showed less lipid stability for retail display life (Juárez and others 2012). Moreover, CLA–GSE-0 treatment showed the lowest TBARS value within treatments without GSE. Ha and others (1990) suggested that CLA may have an antioxidant effect, whereas Fagali and Catalá (2008) and Yu (2001) demonstrated that CLA can provide immediate prevention against free radicals, which would protect against lipid oxidation. Dietary CLA reduced TBARS levels and lipid oxidation of pork loin (Joo and others 2002). Furthermore, direct addition of CLA during the preparation of beef patties decreased TBARS production during refrigerated storage (Chae and others 2004; Hur and others 2004). These findings could demonstrate that CLA reduces the formation of fatty acid free radicals and subsequent oxidation reactions.

In contrast with treatments without GSE that were affected by storage time (P<0.05), GSE treatments were not influenced over time (P>0.05) because of the antioxidant action of GSE, that can delay the formation of TBARS. These findings

are in agreement with previous studies in raw and cooked beef (Ahn and others 2004; Ahn and others 2007; Bañón and others 2007; Rojas and Brewer, 2007). The antioxidant activity of GSE has been associated with the presence of phenolic compounds (Cuppett 2001), whose main mechanism is by acting as free radical scavengers. In the present study, we found that using 250 mg GSE/meat, with 95% of polyphenols, had an antioxidant effect in ground beef enriched with omega-3 and/or CLA and packaged in air for 6 days under retail display conditions. Ahn and others (2002) reported that antioxidant activity of GSE was dependent on the concentration from 0.02% to 0.1% in cooked ground beef. GSE at concentrations as low as 0.1% reduced secondary oxidation products in beef during refrigerated storage (Ahn and others 2007). Rojas and Brewer (2008) compared the antioxidant effect of GSE in beef and pork and concluded that the doses of GSE 0.01% and 0.02% were efficient in both meat species. Furthermore, GSE has also been effective as lipid antioxidant in other meat such as pork (Lorenzo and others 2014) or chicken (Brannan 2008).

3.3. Color

Table 5 shows the effects of beef, GSE and storage time on color parameters (L*, a*, b*, C* and H*) of raw ground beef in aerobic packaging for 6 days under retail display conditions. Although the triple interaction of BxGSExT was not significant (P>0.05), table analysis of it provides valuable information.

L* double interactions B×T (P=0.006), GSE×T (P=0.029) and BxGSE (P=0.057) were analyzed. There were significant differences (P<0.01) on L* values from day 1 according to the beef used, showing C treatments the lowest L* values.

CLA treatments had L* values higher than those of C treatments. Hur and others (2004) reported that CLA addition (0.5 or 2%) increased L* compared to control patties on day 7 in raw refrigerated beef patties. In general, the mean L* value for the Control treatments was lower than those of OME3 and OME3+CLA treatments. Consequently, beef enriched with omega-3 enhanced the ground beef's lightness, which is in agreement with a report by Juárez and others (2012). Regarding the time effect, L* values slightly decreased over time (P<0.001) in control ground beef, whereas in the other treatments L* values in 0 day were similar than those of 6 day (Table 5). No clear data trends were observed by GSE addition, these results are in agreement with those reported by other authors in pork patties (Lorenzo and others 2014).

Double interactions BxGSE, BxT and GSE×T were statistically significant (P<0.05) for a* values. The a* values presented significant differences among treatments according to the beef used. Control beef showed the lowest a* values until day 3. CLA addition resulted in a* values higher than those of Control treatments (P<0.05) until day 3 of refrigerated storage. The addition of 0.5 and 2% of CLA increased a* values in beef patties (Hur and others 2004), whereas a* was not altered when 2 and 4% of CLA were added directly in beef patties (Chae and others 2004). Likewise, omega-3 treatments had a* values higher than those of Control treatments (P<0.05) until day 3. All treatments showed a significant (P<0.001) decrease in redness (around 49%) due to the oxidation of pigments during refrigeration of meat products. GSE addition had a significant effect (P<0.05) on a* of ground beef at day 1, resulting higher values for a* in the GSE treatments. GSE addition (0.01 and 0.02%) did not change measures of redness in beef patties (Rojas and Brewer 2007).

The b* significant interactions (P<0.01) of B×T, GSE×T and BxGSE were studied. There were significant differences (P<0.01) on b* values according to the beef used. The mean b* values of CLA treatments were higher than those of Control treatments (P<0.05) to day 3 of display. However, when 2 and 4% of CLA were added directly in beef patties, the yellowness was not altered (Chae and others 2004). In general, b* values in omega-3 enriched beef were similar those of control beef. The yellowness was mainly affected by storage time (P<0.001) and the b* values decrease around 31% in the treatments. In general, the b* values were not influenced by the addition of GSE (P>0.05), as well as the results found in other studies (Rojas and Brewer 2007).

The interactions of BxGSE, BxT and GSE×T were statistically significant (P<0.001) for C*. Moreover, only the significant interaction of BxT (P=0.001) was found for H*. There were not clear differences among treatments for C* and H* values during display. In general, there was a decrease in C* (P<0.001) while H* increased (P<0.05) over time. These color changes are normally associated with the loss of redness (Bañón and others 2007) and the loss of stability of the color in meat that can result in undesirable color for the consumer. In general, C* and H* were not influenced by the addition of 250 mg GSE/kg meat (P>0.05) during display of the ground beef packaged in air. However, differences for C*and H* were found in beef patties with 100 SO_2+300 GSE (mg /kg meat) compared to patties without additives (Bañón and others 2007).

The relative percentages of MMb measured at the surface of the ground beef during 6 days of display are shown in Fig. 1. There was significant BxGSExT interaction (P<0.001) for percentage of MMb of raw ground beef during display.

There were significant differences (P<0.001) on MMb percentages according to the beef used. The CLA treatments had the lowest % MMb to day 3 of the display. CLA sources for fat improved the oxymyoglobin stability due to the antioxidant effect of CLA (Hur and others 2004). In contrast, the OME treatments had the highest MMb percentages because the color change is partially due to increased lipid oxidation associated with unsaturated fatty acids. But also, there are other factors such as grinding, light and salt which promote the oxidation of pigments and for that, in all treatments MMb gradually increased during the 6 days of display (P<0.001). In addition, in the present study the samples were in aerobic packaged (PVC) which promoted the myoglobin exposure to O_2 and development of aerobic microorganisms and, consequently, resulting the discoloration of the beef. Lavieri and Williams (2014), despite using three types of packaging (vacuum, PVC and MAP) in ground beef, observed an increase of the psychrotrophic bacterias counts which led to discoloration in the ground beef for the refrigerated storage. GSE addition had a significant effect (P<0.01) on % MMb of ground beef at day 3, resulting lower values for % MMb in the GSE treatments, except in the OME3 treatment where the GSE was not able to decrease the MMb. Furthermore, GSE decreased the % MMb in control beef at day 6 (85.45 *vs* 70.81), resulting similar values to those of CLA enriched beef (CLA and OME3+CLA treatments with or without GSE, 70.13%) for that day. GSE was not able to delay the color deterioration in the OME3 enriched beef over time, and the OME3 treatments had 85.51% of MMb at day 6. Therefore, in ground beef it would be necessary to apply the hurdle technology by using natural antioxidant such as GSE, as well as additives and modified atmosphere or vacuum packaging, to protect fully these meat products from the discoloration and microbial

growth. For instance, Bañón and others (2007) reported differences of MMb in beef patties with 100 SO_2+300 GSE (mg /kg meat) compared to control beef (without additives), because the sulphite delayed the color deterioration. Moreover, the combined use of antioxidants and modified atmosphere packaging for meat increase the shelf life of fresh meat (Lorenzo and others 2014; Sánchez-Escalante and others 2003).

It should be noted that differences in methods to enrich the beef with omega-3 and CLA, grinding, salt, color of GSE, different ratios lean/fat, light, storage time and packaging system used, make it difficult to compare these results to those obtained by other authors. For instance, as result of omega-3 and CLA supplementation in diet compared to omega-3 and CLA directly added to meat, these fatty acids are located at different positions and some functions could be different. The grinding of meat destroys aerobic system which may partially explain the accelerated oxidation of the pigment in the ground beef compared to the whole muscle (Honikel 2004). Salt promotes lipid oxidation in raw and cooked meat and accelerates metmyoglobin formation and discoloration in raw meat (Rhee 1999).

3.4. Sensory analysis

3.4.1. Raw ground beef sensory analysis

Evolution of color and odor of the raw ground beef in aerobic packaging for 6 days under retail display conditions are shown in Fig. 2 and Fig. 3 respectively. There was a significant interaction BxGSExT (P<0.001) for odor whereas for the color it was not (P>0.05).

Beef had no significant effect on color during display, except at day 3 (Fig. 2). In that day, the ground beef enriched with CLA had better scores of color than the other treatments (5.59 *vs* 7.47), which could mean that CLA had a positive effect on the color and would support the instrumental color results (Table 5). Moreover, the scores of color in ground beef enriched with omega-3 were similar ($P>0.05$) to those of control beef during the 6 days of display. Color values of treatments increased gradually during storage from 1.99 to 10.38 ($P<0.001$). These results correspond to the discoloration of the beef which is due to the lipid oxidation and the oxidation of pigments, as it has been previously explained in instrumental color results. The GSE did not significantly influence on color except at day 0, resulting higher values in the GSE treatments (1.88 *vs* 2.09, $P<0.05$). Furthermore, it should be noted that GSE addition improved the color value of OME3+CLA treatment at day 3. Rojas and Brewer (2008) did not find differences for visual color when 0.02% of GSE was added to beef that was frozen during 4 months.

Similar to the sensory color, odor was not affected by beef during display, except at day 3 (Fig. 3), when CLA improved the odor values compared to the other beef (5.14 *vs* 7.04, $P<0.001$). These results could mean that CLA had a positive effect on the odor at day 3. Moreover, omega-3 addition did not affected on odor values of ground beef during the 6 days. Odor values of treatments increased over time from 1.90 to 9.49 ($P<0.001$). The development of off-odors might be explained by the secondary products of the lipid oxidation that happens during refrigerated storage (Jongberg and others 2011) and the spoilage of the beef due to microbial populations which lead to the formation of microbial slime formation, off-odor and discoloration (Lavieri and Williams 2014). It should be noted that no slime formation

was observed on the surface of the ground beef in the present study. GSE addition improved the odor value of OME3+CLA treatment at day 3 (7.15 *vs* 4.51, $P<0.001$) and that of OME3 treatment at day 6 (10.74 *vs* 8.77, $P<0.01$). Rojas and Brewer 2008 reported that the addition of 0.02% GSE in beef did not affect odor described as raw meat, grassy, herbal, acid and sweaty.

In the present study, odor and color deteriorated similarly over the time of display. The scores in both sensory parameters were higher than acceptability value (7.5 cm) from day 3, so that the shelf life would be established in 3 days for these raw ground beef packaged in PVC and stored under retail display conditions, results which support those obtained by Lavieri and Williams (2014).

3.4.2. Cooked ground beef sensory analysis

Fig. 4 shows the color and odor values of cooked ground beef at day 2. No significant interaction of BxGSE was found ($P>0.05$) for both sensory parameters. All treatments had color and odor values lower than acceptability value (7.5 cm), so all of them were sensorially acceptable.

Type of beef had significant effect on color ($P<0.05$). Control treatments were the best evaluated, as well as OME3+CLA treatments. However, individual enrichment of omega-3 or CLA in cooked beef did not improve the scores of color compared to control beef. GSE addition did not significantly affect the color of cooked ground beef. These results are in agreement with those reported in other studies about cooked beef during refrigerated storage (Bañón and others 2007; Jongberg and others 2011).

Beef significantly affected odor (*P*=0.016) of cooked ground beef. Control treatments were the best evaluated compared to the other treatments. In the present study warmed over flavor (WOF) were not detected in cooked ground beef at 2 day, although commonly they are developed within 1 to 3 days of refrigerated storage. The odor parameter in GSE treatments was better evaluated than in treatments without GSE (*P*=0.003). Rojas and Brewer (2007) reported that beef patties with 0.02% of GSE had better scores about wet cardboard and rancidity parameters compared to control patties. These findings might justify that GSE can have potential to control some of the negative sensory characteristics associated with unpleasant flavors. However, Bañón and others (2007) did not find significant changes in the odor of cooked beef patties with GSE and low concentrations of sulfite. The different results among the authors could be explained by the presence of additives, packaging type, lipid composition of beef and the content of polyphenolic compounds of GSE used in each of the studies.

3.5. Principal components analysis

Table 6 shows the correlation coefficients among the variables studied in the raw ground beef (TBARS, pH, L*, a*, b*, C*, H*, % MMb, color and odor). The color coordinates a*, b* and C* were positively correlated to each other, and negatively correlated with H* and % MMb. L* was only negatively correlated with the pH. Moreover, color and odor had high correlation coefficient to each other. Likewise, the correlation between a* and b* with sensory parameters was negative and high. TBARS was negatively correlated with a*, b* and C*, and positively correlated with H*, % MMb and odor.

Principal component analysis (PCA) showed that about 94.30% of variability was explained by three main principal components, and 70.94% of it was accounted for the principal component 1 (PC1). The increase in metmyoglobin, H* and color and odor and the decrease in a*, b* and C* clearly reflect a degradation in ground beef quality; hence, PC1 was a ground beef quality degradation factor. The principal component 2 (PC2, 16.65%) were formed by L* and pH. Principal component 3 (PC3, 6.71%) was formed by TBARS, so the PC3 was a ground beef lipid oxidation factor.

When plotting the treatments of raw ground beef for 0 and 6 days on the same bi-dimensional space, a clear separation was observed by PC1 (Fig. 5). Treatments at day 0 were placed on the left side, whereas treatments at 6 day were placed on the right side. Consequently, the variables H*, % MMb, color and odor increased with increasing storage time, whereas a*, b* and C* decreased, so they can be used as indicators of the loss of raw ground beef quality. Likewise, Insausti and others (2008) reported a clear separation by factor 1, related with the beef quality degradation, when plotting days of storage on the same bi-dimensional space.

Moreover at 6 day, PC3 separated GSE treatments (negative side of PC3) from the treatments without GSE (positive side of PC3). The PC3 was related with TBARS, so the lipid oxidation can be used as indicator of the effectiveness of GSE in raw ground beef at 6 day of display. The PCA also separated sausages with GSE from the control group (without antioxidants) by factor 1, which was positively related with moisture content, aw, color parameters and acetic concentration and inversely related with TBARS and TPA (Lorenzo and others 2013). In the present study, it should be noted that CLA treatment without GSE (CLA–GSE-0) was placed

at negative side of PC3 because CLA had antioxidant effect, such as was explained in TBARS section. Likewise, OME3 treatments were at the top of positive side of PC3 as they presented the highest level of oxidation due to their enrichment with omega-3 fatty acids. Therefore, the PC3 could differentiate the ground beef without added GSE according the enrichment or no with omega-3 and/or CLA, because PC3 was related with the oxidative stability which depended on lipid composition of the ground beef.

4. Conclusions

The enrichment of beef with omega-3 and CLA improves the lipid profile of the beef, although the oxidative stability is impaired. The enrichment of omega-3 and omega-3 plus CLA by modifying the diet of bulls have not been enough to cause variations on the instrumental color neither the sensory parameters, so, the visual appearance of enriched beef is similar to conventional beef. The results pointed to the potential value of CLA enrichment to stabilize the lipid oxidation. Furthermore, the color in beef enriched with CLA was improved until day 3, which would be interesting to obtain more attractive products for the consumers. According to the sensory analyses, the shelf life of the ground enriched beef would be established in 3 days, under aerobic packaging and retail display conditions.

GSE addition prevented rancidity in ground raw ground beef enriched with omega-3 and/or CLA and did not affect the instrumental color neither the sensory parameters in ground beef. The results suggest that GSE can be a technologically viable alternative for stabilizing the lipid oxidation in new fresh meat products,

although it should be used in conjunction the hurdle technologies to reduce the discoloration and the microbial growth.

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Table 1–Proximate and fatty acid composition of ground raw beef enriched with omega-3 and/or CLA.

Beef [a]	**C**	**OME3**	**CLA**	**OME3+CLA**
Proximate composition (%)				
Moisture (%)	69.67	69.54	68.61	69.88
Protein (%)	21.66	21.66	21.13	21.65
Fat (%)	5.05	4.86	5.37	4.63
Fatty acid profile (% total fatty acid)				
Saturated Fatty Acid	42.51	37.84	39.68	38.98
Monounsaturated Fatty Acid	54.84	58.17	57.48	56.49
Polyunsaturated Fatty Acid	2.65	3.98	2.84	4.53
Fatty acid content (mg FA /100 g ground beef)				
Omega-3	1.46	5.87	1.70	6.39
CLA	9.16	7.78	12.56	11.20

[a] Beef from animals fed different diets: C, conventional; OME3, omega-3; CLA, conjugated linoleic acid; OME3+CLA, omega-3 plus CLA.

Table 2–Experimental design.

Treatments	Beef	mg GSE/kg meat
C–GSE-0	C	0
OME3–GSE-0	OME3	0
CLA–GSE-0	CLA	0
OME3+CLA–GSE-0	OME3+CLA	0
C–GSE-250	C	250
OME3–GSE-250	OME3	250
CLA–GSE-250	CLA	250
OME3+CLA–GSE-250	OME3+CLA	250

General formulation: 98% ground beef + 2% salt.
GSE used: GSE-0, without GSE; GSE-250, 250 mg GSE/kg meat.
Beef from animals fed different diets: C, conventional; OME3, omega-3; CLA, conjugated linoleic acid; OME3+CLA, omega-3 plus CLA.

Table 3–Evolution of pH values in raw ground beef enriched with omega-3 and/or CLA stored in aerobic packaging for 0 and 6 days under retail display conditions.

GSE [a]	GSE-0				GSE-250					*P*-value		
Beef [b]	C	OME3	CLA	OME3+CLA	C	OME3	CLA	OME3+CLA	SEM	B	GSE	BxGSE
Days												
0	5.33 [a]	5.26 [bc]	5.29 [abc]	5.30 [ab]	5.32 [a]	5.27 [abc]	5.31 [ab]	5.24 [c]	0.012	<0.001	0.430	0.017
6	5.32	5.23	5.18	5.17	5.30	5.22	5.17	5.15	0.059	0.045	0.405	0.823
SEM	0.039	0.066	0.052	0.037	0.019	0.017	0.053	0.034				
P-value	0.863	0.498	0.188	0.048	0.487	0.071	0.114	0.131				

[a] GSE used: GSE-0, without GSE; GSE-250, 250 mg GSE/kg meat.
[b] Beef from animals fed different diets: C, conventional; OME3, omega-3; CLA, conjugated linoleic acid; OME3+CLA, omega-3 plus CLA. Mean values in the same row (different treatments on the same storage day) with different letter presented significant differences ($P<0.05$). SEM: Standard Error of Mean.

Table 4–Evolution of thiobarbituric acid reagent substances (TBARS, mg MDA/kg meat) in raw ground beef enriched with omega-3 and/or CLA stored in aerobic packaging for 0 and 6 days under retail display conditions.

GSE [a]	**GSE-0**				**GSE-250**					***P*-value**		
Beef [b]	**C**	**OME3**	**CLA**	**OME3+CLA**	**C**	**OME3**	**CLA**	**OME3+CLA**	**SEM**	**B**	**GSE**	**BxGSE**
Days												
0	0.51	0.62	0.44	0.72	0.49	0.49	0.36	0.63	0.133	0.241	0.400	0.982
6	2.38 [b]	4.59 [a]	1.56 [c]	4.42 [a]	0.47 [d]	0.61 [d]	0.48 [d]	0.70 [d]	0.156	<0.001	<0.001	<0.001
SEM	0.100	0.151	0.247	0.081	0.124	0.173	0.117	0.091				
P-value	<0.001	<0.001	0.018	<0.001	0.928	0.650	0.483	0.584				

[a] GSE used: GSE-0, without GSE; GSE-250, 250 mg GSE/kg meat.
[b] Beef from animals fed different diets: C, conventional; OME3, omega-3; CLA, conjugated linoleic acid; OME3+CLA, omega-3 plus CLA. Mean values in the same row (different treatments on the same storage day) with different letter presented significant differences ($P<0.05$). SEM: Standard Error of Mean.

Table 5–Evolution of Lightness (L*), Redness (a*), Yellowness (b*), Chroma (C*) and Hue angle (H*) in the raw ground beef enriched with omega-3 and/or CLA stored in aerobic packaging for 0, 1, 3 and 6 days under retail display conditions.

GSE [a]		GSE-0				GSE-250					*P*-value		
Beef [b]		C	OME3	CLA	OME3+ CLA	C	OME3	CLA	OME3+ CLA	SEM	B	GSE	BxGSE
	Days												
L*	0	39.11 ^{1}	39.51	39.82	38.13 ^{12}	38.66 ^{1}	39.84	40.02 ^{12}	41.13 ^{1}	0.824	0.626	0.190	0.171
	1	36.25 ^{12}	36.89	38.11	37.41 ^{12}	37.87 ^{12}	38.95	40.57 ^{1}	40.20 ^{1}	0.634	0.003	<0.001	0.813
	3	33.65 ^{2}	36.50	38.90	36.47 ^{2}	34.29 ^{3}	37.90	37.48 ^{2}	36.38 ^{2}	0.679	<0.001	0.786	0.207
	6	37.57 ^{bc1}	38.30 ^{bc}	39.27 ^{ab}	39.62 ^{ab1}	36.08 ^{c23}	39.00 ^{abc}	41.61 ^{a1}	42.07 ^{a1}	0.704	<0.001	0.049	0.022
	SEM	0.814	0.859	0.577	0.741	0.672	0.554	0.791	0.639				
	P-value	<0.001	0.069	0.221	0.034	<0.001	0.122	0.006	<0.001				
a*	0	14.23 ^{1}	15.30 ^{1}	15.66 ^{1}	13.94 ^{1}	13.31 ^{1}	15.13 ^{1}	15.81 ^{1}	15.29 ^{1}	0.743	0.061	0.847	0.494
	1	8.57 ^{2}	10.12 ^{2}	10.49 ^{2}	9.17 ^{2}	9.72 ^{2}	11.71 ^{2}	12.35 ^{2}	11.43 ^{2}	0.413	<0.001	<0.001	0.597
	3	6.70 ^{2}	8.86 ^{2}	9.66 ^{23}	8.81 ^{2}	7.10 ^{3}	7.89 ^{3}	10.65 ^{2}	10.07 ^{23}	0.442	<0.001	0.201	0.065
	6	8.30 ^{ab2}	6.49 ^{d3}	7.98 ^{abcd3}	7.72 ^{abcd2}	6.74 ^{cd3}	8.02 ^{abc3}	6.93 ^{bcd3}	8.58 ^{a3}	0.346	0.067	0.830	<0.001
	SEM	0.540	0.506	0.593	0.463	0.418	0.515	0.534	0.488				
	P-value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001				
b*	0	10.27 ^{1}	10.50 ^{1}	11.91 ^{1}	10.68 ^{1}	9.36 ^{1}	11.17 ^{1}	12.34 ^{1}	12.16 ^{1}	0.507	<0.001	0.248	0.133
	1	7.38 ^{c2}	7.76 ^{c2}	8.98 ^{abc2}	7.78 ^{c2}	7.61 ^{c2}	8.31 ^{bc2}	9.94 ^{ab2}	10.30 ^{a2}	0.381	<0.001	<0.001	0.018
	3	8.11 ^{abc2}	7.45 ^{bc2}	9.51 ^{a2}	7.80 ^{abc2}	6.56 ^{c2}	6.51 ^{c3}	9.20 ^{ab2}	9.34 ^{ab2}	0.441	<0.001	0.315	0.005
	6	8.52 ^{a2}	7.15 ^{ab2}	8.45 ^{a2}	6.66 ^{b2}	6.90 ^{ab2}	7.87 ^{ab23}	7.30 ^{ab3}	7.43 ^{ab3}	0.392	0.357	0.393	0.001
	SEM	0.391	0.526	0.463	0.476	0.390	0.457	0.400	0.331				
	P-value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001				

GSE [a]		GSE-0				GSE-250					*P*-value		
Beef [b]		C	OME3	CLA	OME3+ CLA	C	OME3	CLA	OME3+ CLA	SEM	B	GSE	BxGSE
	Days												
C*	0	17.65 ^{1}	18.58 ^{1}	19.79 ^{1}	17.70 ^{1}	16.34 ^{1}	18.84 ^{1}	20.14 ^{1}	19.58 ^{1}	0.701	0.001	0.554	0.170
	1	11.33 ^{2}	12.78 ^{2}	13.86 ^{2}	12.05 ^{2}	12.39 ^{2}	14.36 ^{2}	15.86 ^{2}	15.40 ^{2}	0.496	<0.001	<0.001	0.128
	3	10.59 ^{c2}	11.60 ^{bc23}	13.70 ^{ab2}	11.79 ^{bc2}	9.70 ^{c3}	10.29 ^{c3}	14.12 ^{a2}	13.78 ^{ab2}	0.507	<0.001	0.892	0.008
	6	11.93 ^{a2}	9.74 ^{c3}	11.69 ^{ab3}	10.20 ^{bc2}	9.71 ^{c3}	11.25 ^{abc3}	10.18 ^{bc3}	11.65 ^{ab3}	0.360	0.584	0.441	<0.001
	SEM	0.545	0.652	0.480	0.523	0.429	0.623	0.491	0.457				
	P-value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001				
H*	0	36.22 ^{3}	34.40 ^{2}	37.52 ^{2}	37.62	35.19 ^{3}	36.46 ^{2}	38.17 ^{2}	38.68	1.761	0.285	0.583	0.849
	1	40.91 ^{23}	37.14 ^{2}	40.76 ^{12}	40.12	37.99 ^{23}	35.43 ^{2}	38.76 ^{2}	41.94	1.173	0.001	0.152	0.205
	3	50.17 ^{a1}	39.67 ^{b2}	44.62 ^{ab12}	41.20 ^{b}	42.72 ^{ab12}	39.06 ^{b2}	40.91 ^{b12}	43.32 ^{ab}	1.701	0.001	0.048	0.040
	6	45.49 ^{12}	47.53 ^{1}	46.54 ^{1}	40.58	45.65 ^{1}	44.39 ^{1}	46.60 ^{1}	42.07	2.037	0.049	0.805	0.707
	SEM	1.546	1.610	2.228	1.532	1.740	1.324	1.890	1.550				
	P-value	<0.001	<0.001	0.033	0.381	0.001	<0.001	0.013	0.199				

[a] GSE used: GSE-0, without GSE; GSE-250, 250 mg GSE/kg meat.
[b] Beef from animals fed different diets: C, conventional; OME3, omega-3; CLA, conjugated linoleic acid; OME3+CLA, omega-3 plus CLA.
Mean values in the same row (different treatments on the same storage day) with different letter presented significant differences (P<0.05).
Mean values in the same column (same treatment in different days) with different number presented significant differences (P<0.05).
SEM: Standard Error of Mean.

Table 6–Pearson's correlation coefficient among response variables: TBARS, pH, L*, a*, b*, C*, H*, % MMb, color and odor.

	TBARS	pH	L*	a*	b*	C*	H*	% MMb	Odor	Color
TBARS	-									
pH	-0.051	-								
L*	-0.204	**-0.635****	-							
a*	**-0.528***	0.401	0.207	-						
b*	**-0.520***	0.406	0.205	**0.952****	-					
C*	**-0.535***	0.405	0.214	**0.996****	**0.976****	-				
H*	**0.426**	-0.300	-0.173	**-0.900****	**-0.730****	**-0.857****	-			
% MMb	**0.520***	-0.414	-0.182	**-0.956****	**-0.887****	**-0.946****	**0.887****	-		
Odor	**0.554***	-0.485	-0.072	**-0.964****	**-0.895****	**-0.951****	**0.897****	**0.966****	-	
Color	0.496	-0.475	-0.130	**-0.975****	**-0.903****	**-0.963****	**0.905****	**0.980****	**0.986****	-

* Significance at level $P<0.05$.
** Significance at level $P<0.01$.

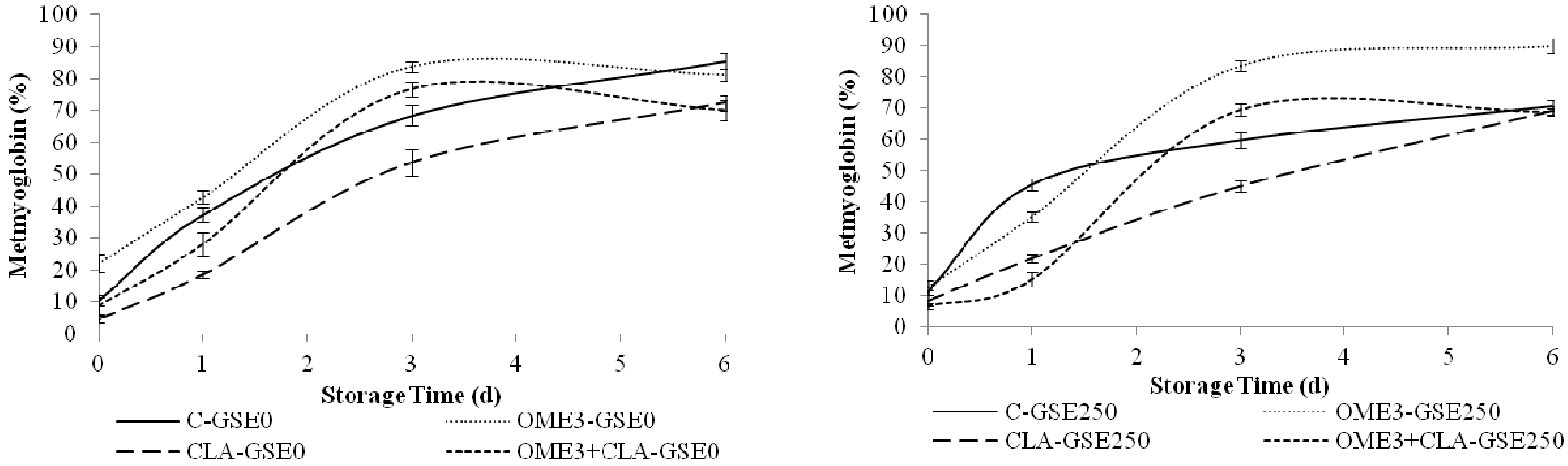

Figure 1–Evolution in the percentage of surface metmyoglobin (means ± SE) in the raw ground beef enriched with omega-3 and/or CLA stored in aerobic packaging for 0, 1, 3 and 6 days under retail display conditions.
GSE used: GSE-0, without GSE; GSE-250, 250 mg GSE/kg meat.
Beef from animals fed different diets: C, conventional; OME3, omega-3; CLA, conjugated linoleic acid; OME3+CLA, omega-3 plus CLA.

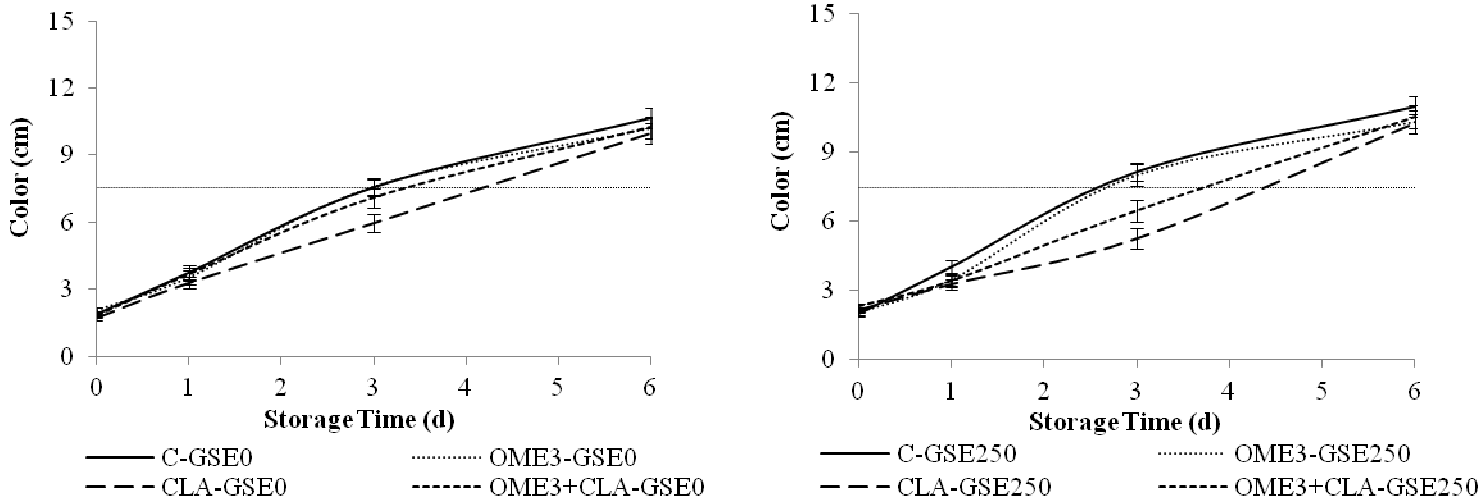

Figure 2–Sensory evaluation: color of the raw ground beef enriched with omega-3 and/or CLA stored in aerobic packaging for 0, 1, 3 and 6 days under retail display conditions (15 cm: maximal discoloration scores; 7.5 cm: acceptability limit).
GSE used: GSE-0, without GSE; GSE-250, 250 mg GSE/kg meat.
Beef from animals fed different diets: C, conventional; OME3, omega-3; CLA, conjugated linoleic acid; OME3+CLA, omega-3 plus CLA.

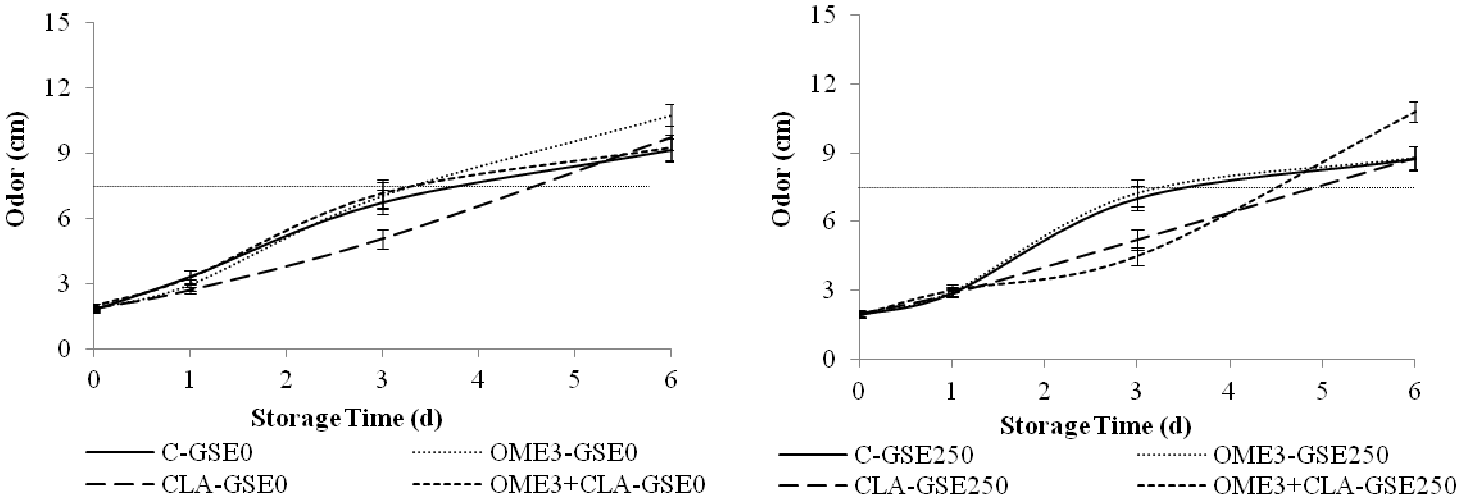

Figure 3–Sensory evaluation: odor of the raw ground beef enriched with omega-3 and/or CLA stored in aerobic packaging for 0, 1, 3 and 6 days under retail display conditions (15 cm: maximal odor scores; 7.5 cm: acceptability limit).
GSE used: GSE-0, without GSE; GSE-250, 250 mg GSE/kg meat.
Beef from animals fed different diets: C, conventional; OME3, omega-3; CLA, conjugated linoleic acid; OME3+CLA, omega-3 plus CLA.

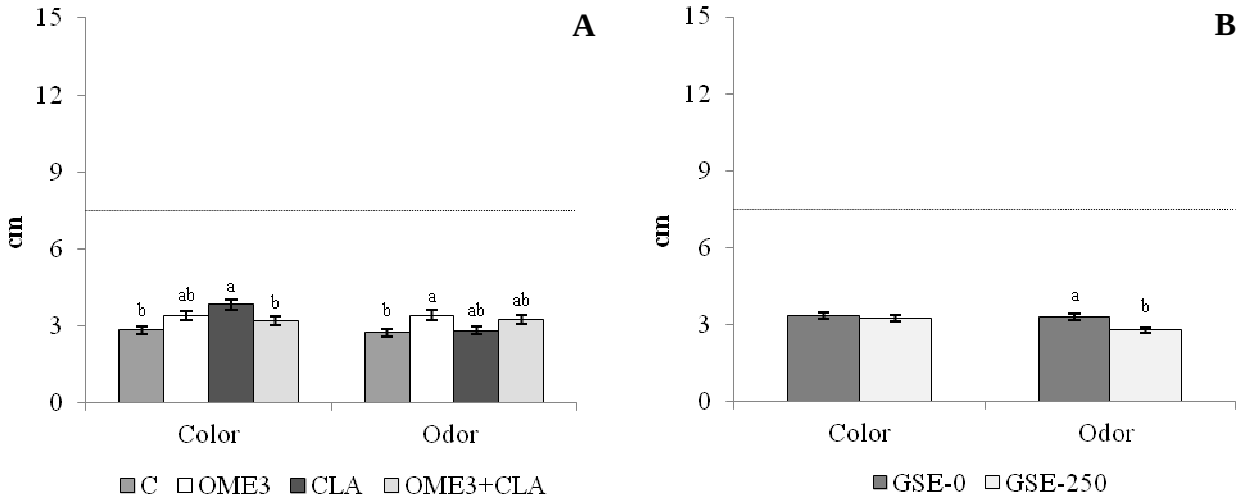

Figure 4–Sensory evaluation of color and odor (means ± SE) for the ground beef enriched with omega-3 and/or CLA, cooked at day 2 (15 cm: maximal discoloration and odor scores; 7.5 cm: acceptability limit). Effect of beef from animals fed different diets: C, conventional; OME3, omega-3; CLA, conjugated linoleic acid; OME3+CLA, omega-3 plus CLA (A). Effect of GSE used: GSE-0, without GSE; GSE-250, 250 mg GSE/kg meat (B).

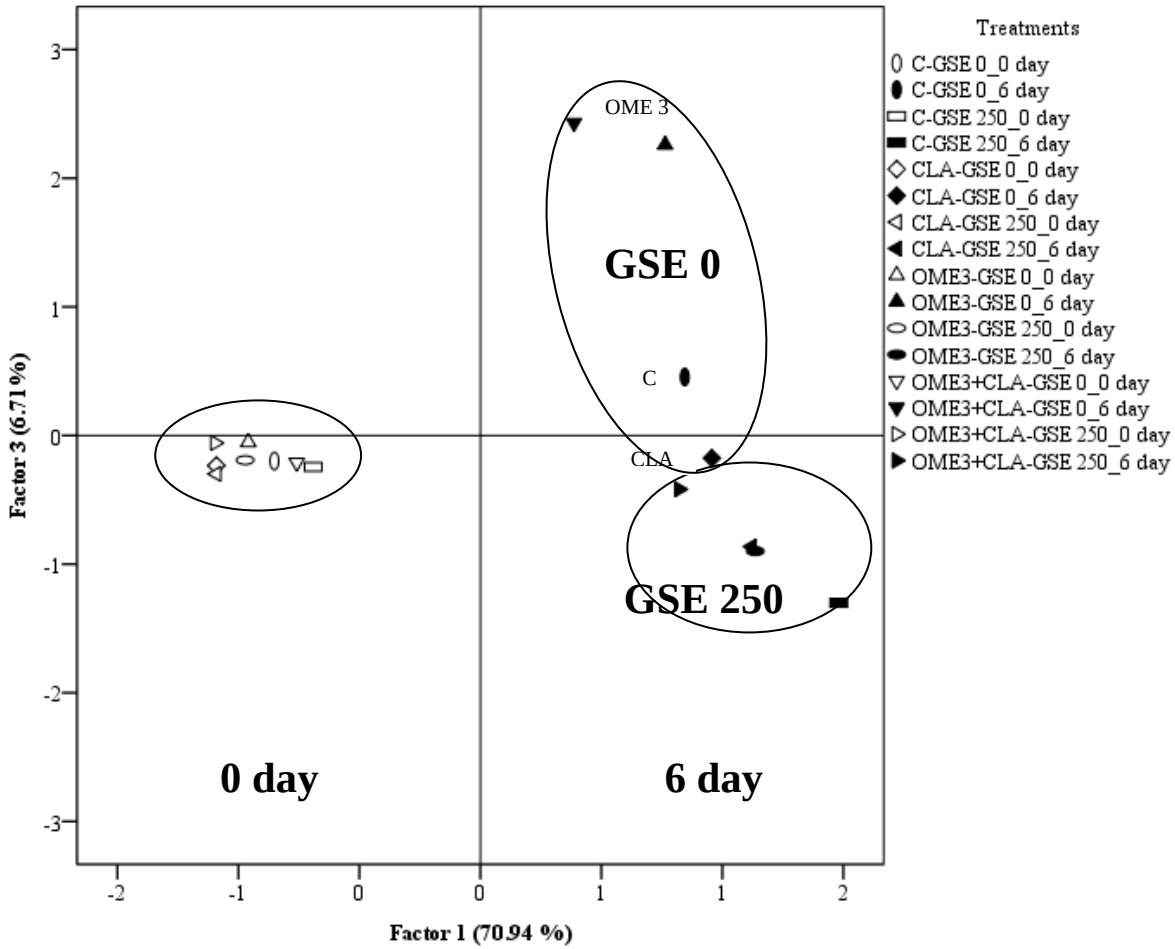

Figure 5–Plot of treatments of raw ground beef for 0 and 6 days on the bi-dimensional space formed by factors 1 and 3 obtained by principal component analysis of TBARS, pH, L*, a*, b*, C*, H*, % MMb, color and odor variables.
GSE used: GSE-0, without GSE; GSE-250, 250 mg GSE/kg meat.
Beef from animals fed different diets: C, conventional; OME3, omega-3; CLA, conjugated linoleic acid; OME3+CLA, omega-3 plus CLA.

Shelf life of low-fat beef patties enriched with omega-3, CLA and oleic fatty acids and influence of grape seed extract

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ABSTRACT

The shelf life and oxidative stability of refrigerated raw ground beef enriched with omega-3 and/or CLA was studied. Grape seed extract (GSE) was used to inhibit the lipid oxidation in the ground beef. Eight treatments of ground beef were established according to the enrichment of beef (control, enriched with omega-3, with CLA, or with omega-3 plus CLA) and the use of GSE (0 and 250 mg GSE/kg product). Fresh beef was ground and mixed with GSE and salt. Treatments of beef were stored at 2 ± 1 ºC in aerobic packaging for 0, 1, 3 and 6 days under retail display conditions. Oxidation stability (TBARS), pH, instrumental colour, metmyoglobin formation and sensory attributes (colour and odour) were measured. Omega-3 enriched beef increased the oxidation level at day 6 as determined by TBARS ($P<0.05$) but the instrumental colour was not affected. The enrichment of CLA improved the coordinates of colour ($P<0.05$) until day 3 and decreased the oxidation at day 6 ($P<0.05$). There were not differences for colour and odour values among type of beef during display, except at day 3, when CLA treatments were the best valuated. Addition of GSE decreased the oxidation level ($P<0.001$) and did not affect the instrumental colour neither the sensory parameters.

Key words: beef patties, omega-3, CLA, grape seed extract, shelf life, olive oil

Introduction

There is increasing evidence to indicate that relation between nutrition and human health and public health policies have recommended population to decrease in the consumption of fat, saturated fatty acids and to increase the intake of polyunsaturated fatty acids (WHO, 2003). The n-3 PUFAs ALA, EPA and DHA provide a wide range of benefits from general improvements in health to protection against inflammation and disease (Ganesan et al., 2014). Likewise, CLA has been associated with potential benefits in some diseases such as cancer, atherosclerosis, growth, obesity, osteoporosis, and immune responses (Pariza, 2004; Park, 2009; Dilzer and Park, 2012). Moreover, a recent study has associated saturated fat with cardiovascular disease (Siri-Tarino et al., 2010).

In general, meat is considered an important component of the diet by consumers (Verbeke, Pérez-Cueto, Barcellos, Krystallis, & Grunert, 2010). Dietary factors have a bigger influence than genetic factor on the fatty acid composition of beef (De Smet et al., 2004). The supplementation of ruminant diets with n-3 and CLA would be the most effective approach to decrease SFA and to increase the n-3 and CLA FA. However, the enrichment with PUFA and technological operations, such as the minced or addition of salt, can accelerate oxidative processes giving rise to the deterioration of the flavour, colour, texture, thus limiting the shelf life of these products. However, the use of antioxidants can overcome these problems.

Grape seed extracts (GSE) are natural antioxidants that, in addition to its antioxidant properties, have specific biological activities that provide beneficial and healthy effects for the human body (Gharras, 2009). GSE have been widely used at low concentrations (0.01–1%) in various food and beverage and has demonstrated antioxidant and antimicrobial activities alone or in combination with other hurdle technologies in various food applications such as tomatoes, frankfurters, raw and cooked meat, poultry products and fish (Perumalla et al., 2011). Gómez et al. (2014) reported that dose of 250 mg GSE / kg meat was enough to inhibit the lipid oxidation in ground beef enriched with omega 3 and/or CLA.

The fat is necessary in meat products because it helps provide the desired taste characteristics associated with beef. When the amount of marbling increases above 3%, there is a slight increase in palatability with further thresholds occurring at 5% and 7%

(Savell and Cross, 1988). One of the alternatives would be to add olive oil due to its healthy properties (Ruiz-Canela & Martínez-González, 2011; Covas, Konstantinidou, & Fito, 2009). Several authors have experimented with the incorporation of olive oil in meat products such as sausage or frankfurters (Ansorena & Astiasarán, 2004a, 2004b; Beriain et al., 2011; Bloukas et al., 1997; Muguerza et al., 2002; Muguerza et al., 2001; Severino et al., 2003). There have been fewer studies where the olive oil has been stabilized in an oil-in-water emulsion and has added to beef patties (López-López et al., 2010, 2011; Martínez et al., 2012).

There have been found no studies assaying processes for reformulation of healthier beef patties based on beef enriched with n-3 and CLA from bulls fed diets enriched with these FAs, the incorporation of olive oil in water emulsion and GSE analyzing their properties during refrigerated retail display. Thus, the aim of the present study was to examine the shelf life and oxidative stability in refrigerated raw beef patties enriched with omega-3 and/or CLA and oleic fatty acids. Grape seed extract was also used as natural antioxidant to inhibit the lipid oxidation in the beef patties enriched with unsaturated fatty acids.

2. Material and methods

2.1. Materials

2.1.1. Beef

Beef loin cuts were obtained at 24 h *postmortem* from the right carcass side of 48 Holstein entire males (10.7 months old) fed with one of four dietary treatments. All animal diets had similar composition but differed in the content of whole linseed and conjugated linoleic acid (CLA): Control (C, conventional commercial ration, 0% linseed and 0% CLA), omega-3 (OME3, conventional ration enriched with omega-3 fatty acids through the addition of 10% linseed), CLA (CLA, conventional ration enriched with CLA through the addition of 2% CLA), and omega-3+CLA (OME3+CLA, conventional ration enriched with omega-3 and CLA fatty acids through the addition of 10% linseed plus 2% CLA). Animal productive performance and carcass characteristics of these animals were reported by Albertí et al. (2013). Animals were slaughtered with an average live weight of 458.4 ± 16.6 kg at an EU-licensed commercial abattoir following standard procedures. Vacuum packaged loin cuts were transported to the Public University of Navarre meat laboratory and they were stored at

–18 ºC until required for the experiment. The fatty acid composition of loin cuts are shown in Table 1.

2.1.2. Extract

A commercial grape seed extract (GSE) with a polyphenol content of 95% was used. GSE was provided by Exxentia (Madrid, Spain) and it was a water soluble homogeneous brown powder. The use of GSE (GSE-0 and GSE-250, 0 and 250 mg GSE/kg meat respectively) on ground beef was studied. The selection of the dose used (250 mg GSE/kg meat) was based on a previous study (Gómez et al., 2011).

2.1.3. Other additives

Commercial olive oil (13% SFAs, 79% MUFAs and 8% PUFAs) was obtained from Urzante (Navarra, Spain). Other additives used were common salt (NaCl), and soy protein isolate (SUPRO 545, Sakana Alimentaria S.L., Navarre, Spain).

2.2. Oil-in-water emulsion preparation

Olive oil was used as pre-emulsified fat with soy protein isolate. The mixture was olive oil: soya fiber: water (2: 0.8: 5). The emulsion was prepared by mixing, for 2 min, 5 parts of water with 0.8 part of soy protein isolate and then with 2 parts of the olive oil for another 3 min. These were placed in moulds and stored at 2 ± 1 ºC overnight pending product manufacture.

2.3. Beef patty preparation

Eight treatments (Table 2) of beef patties were established according to beef enriched PUFA (C, OME3, CLA and OME3+CLA) and the use of GSE (GSE-0 and GSE-250): C–GSE-0, OME3–GSE-0, CLA–GSE-0, OME3+CLA–GSE-0, C–GSE-250, OME3–GSE-250, CLA–GSE-250 and OME3+CLA–GSE-250.

The frozen beef loin cuts were allowed to thaw 24 h before being minced. The twelve beef loin cuts from each one of four dietary treatments (C, OME3, CLA, OME3+CLA) were minced together through a Cato mincer (TALSABELL S.A., Sabadell, Spain). The minced beef (C, OME3, CLA, OME3+CLA), salt (2%), GSE (0, 250 mg GSE/kg product) and emulsion were then blended together by a Sammic mixer (Sammic S.L., Azkoitia, Spain) for 60 seconds. The mix was then weighed into portions of 100 g and formed in to patties between grease proof papers using a patty press, to

give average dimensions of 10 cm diameter and 1.5 cm thickness. The meat temperature during processing did not exceed 7 ºC. The patties were placed in transparent plastic trays covered with transparent polyvinyl chloride film (PVC) and stored at 2 ± 1 ºC for 6 days in a display cabinet illuminated (10 hours/day) with white fluorescent light, simulating retail display conditions. On each evaluation day (0, 1, 3 and 6), samples were prepared for proximate composition, pH, colour, TBARS, and sensory analyses.

2.4. Proximate analysis and pH

The protein (AOAC, 1984), total fat (ISO 1443, 1973) and moisture (ISO 1442, 1973) content were determined for each sample. The analyses were performed in duplicate for each sample.

The pH of the treatments at 0 and 6 days of the display were measured (AOAC, 2003) in quadruplicate for each sample. The pH was measured by homogenization in water using a Crison GLP22 pHmeter (Crison Instruments S.A., Barcelona, Spain) equipped with a 6 mm (diameter) penetration probe.

2.5. Colour

Colour was measured using a Minolta CM-2002 spectrophotometer (Konica Minolta Business Technologies Inc., Tokyo, Japan) making five measurements per sample. Colour parameters were evaluated directly on the patties surface during display (0, 1, 3 and 6 days) using the CIE L*a*b* system, illuminant D65 and 10º as the standard observing point. Results were expressed as CIELab values: Lightness (L*), Redness (a*), Yellowness (b*), Chroma (C*) and Hue angle (H*); $C = (a^{*2} + b^{*2})^{0.5}$; H = arctg b* / a*.

2.6. Thiobarbituric acid-reactive substances

Thiobarbituric acid reactive substances (TBARS) values were determined, at the 0 and 6 days of the display, in duplicate for each sample, using the method described by (Tarladgis, Watts, Younathan, & Dugan, 1960). The absorbance was measured at 538 nm in a spectrophotometer (UV-2101PC, Shimadzu, Kyoto, Japan). The TBARS value, expressed as the mg malonaldehyde/kg meat, was obtained using a conversion factor based on a standard curve using 1,1,3,3-tetraethoxypropane (TEP).

2.7. Raw beef patties sensory analysis

A sensory analysis was made on the raw beef patties at the 0, 1, 3 and 6 days of the display. The methodology carried out to assess beef odour and colour degradation was the one described by Gómez et al. (2014, data not published yet). A semi-trained sensory panel of 15 panelists was recruited from Public University of Navarre. Firstly, odour evaluations were performed under soft red light (≈ 100 lux), and then colour evaluations were evaluated under white light (≈ 450 lux). Panellists rated odour and colour using a 15 cm line anchored at each end with the terms: on the left side "non detectable off-odour", "bright fresh red meat" and on the right side "extreme off-odour", "brown, greenish, discoloured meat", respectively. The acceptability limit was anchored in the middle of the line (7.5 cm from each end). The results were quantified by measuring the distance in cm of the panellists mark from the left side.

2.8. Cooked beef patties sensory analysis

Samples were taken from each of the treatments at 2 days of the display. Samples were cooked in a double hot-plate grill pre-heated to 200 ºC until final internal temperature reached 71 ºC that was determined using individual thermocouples inserted into the geometric centre of the meat. Samples were coded and kept warm in a heater for between 5 and 15 min until sensory analyses. The methodology carried out to assess cooked beef patties odour and colour degradation was the one described previously for raw beef patties. Panellists rated odour and colour using a 15 cm line anchored at each end with the terms: on the left side "non detectable off-odour", "typical aspect cooked meat" and on the right side "extreme off-odour", "discoloured cooked meat", respectively. Furthermore, the sensory panel evaluated el aroma característico, flavour característico, jugosidad, cohesividad, granulosidad y overall acceptability. The intensity of every attribute was expressed on a 15 cm line anchored at each end with the terms: the left side "sensation not perceived" and on the right side "maximum of the sensation". During sensory evaluation, the panellists were situated in private cabinets illuminated with soft red light (≈ 100 lux).Water was used to clean the palates and remove residual flavours, at the beginning of the session and in between samples.

2.9. Statistics

Two master batches of patties were manufactured producing two replications of the entire experiment. Data were analyzed using a general linear model (GLM)

procedure (IBM-SPSS version 21 for Windows). The effect of the replicate was not significant between the two batches from each the treatments. For pH, TBARS, instrumental colour and sensory data of raw beef patties, the statistical model included the fixed effects of beef (B), grape seed extract (GSE) and storage time (T), as well as their interactions and residual error. For sensory data of cooked patties, the statistical model included the fixed effects of B and GSE, as well as their interaction and residual error. Differences among means were analyzed by the Tukey's test. Pearson's correlation coefficients between variables were calculated. For all tests, the level of significance was set at $P<0.05$. Multivariate analysis, namely, factor analysis, was used to examine the relationships among all the variables studied. Factors were extracted using the principal component analysis (PCA) method. The Varimax rotation method was applied to the factors to facilitate interpretation and to maximise the explained variance (Morrison, 1990).

3. Results and Discussion

3.1. Proximate composition

The proximate composition of beef patties enriched with omega-3 and/or CLA is given in Table 3. As can be seen, the moisture was around 69%, similar to those other studies (Martínez et al., 2012). The protein content was around 21%, slightly higher than the conventional (16.23%) or modified (19.86%) patties from the study by (Martínez et al., 2012). The fat content was around 7%, it was lower than composition of conventional beef patties from Spain (18.68%, AESAN 2012) and therefore, the beef patties of the present study followed nutritionists' recommendations more closely than conventional products.

3.2. pH

Table 4 shows the pH values of the treatments, whose triple interaction BxGSExT was not significant ($P>0.05$). The interaction BxGSE was statistically significant ($P<0.05$) at 0 day, whereas there were not significance differences ($P>0.05$) on pH values at 6 day. The pH values ranged between 5.46 and 5.76 and any clear trends were not found. The pH values were not affected by time in the beef patties, except the CLA–GSE-0 treatment that had lower pH values at 6 day. GSE supplementation had no significant effect on pH during the display, these results agree

with those obtained by (Bañón, Díaz, Rodríguez, Garrido, & Price, 2007) and (Rojas & Brewer, 2007).

3.3. TBARS

Table 5 shows the level of lipid oxidation of the treatments. There was a significant interaction BxGSExT (P=0.036) for TBARS of beef patties during display. GSE factor as well as the interaction of BxGSE had no significant effects (P>0.05) on lipid oxidation at 0 day, whereas the enrichment of beef affected the TBARS level (P=0.011). Moreover, interaction of BxGSE and beef and GSE factors were statistically significant (P<0.05) at 6 day.

The treatments without GSE increased gradually from 0.49 mg MDA/kg to around 5.40 mg MDA/kg (P<0.001) during display. The omega-3 enriched beef patties had higher oxidation level than the other ones at day 0 (0.67 *vs* 0.42) and at day 6 (6.35 *vs* 5.08) because polyunsaturated fatty acids are more susceptible to the lipid oxidation. Likewise, steaks with higher omega-3 fatty acid content (Juárez et al., 2012) or ground beef enriched with omega-3 (Gómez et al., 2014) showed less lipid stability during the display. Moreover, beef patties enriched with CLA (CLA and OME3+CLA) showed the lowest TBARS value within treatments without GSE at 6 day (5.01 mg MDA/kg). The antioxidant effect of CLA was suggested by (Ha, Storkson, & Pariza, 1990), whereas other studies demonstrated that CLA can provide immediate prevention against free radicals ((Fagali & Catalá, 2008); (Yu, 2001)) which would reduces the subsequent oxidation reactions. In the present study, CLA reduced the TBARS level, as well as it protected against lipid oxidation in other meat products such as pork loin (Joo, Lee, Ha, & Park, 2002), chicken meat (Du, Ahn, Nam, & Sell, 2000) and beef patties ((Chae, Keeton, & Smith, 2004; Hur et al., 2004); Gómez et al., 2014)).

Campo et al. (2006) established the value of 2 mg of malonaldehyde / kg muscle to be considered the upper limit of rancidity for the acceptability of beef consumer. TBARS values from GSE patties remained below this threshold during the whole retail display, while TBARS values in patties without GSE went beyond this value by day 6. It can be explained by the antioxidant action of GSE, which can delay the TBARS formation. The phenolic compounds act as free radical scavengers, so the antioxidant

activity of GSE has been associated with the presence of these compounds (Cuppett, 2001). Several authors have also reported antioxidant activity of GSE in raw ground beef ((Juhee Ahn, Grün, & Mustapha, 2004; Bañón et al., 2007); (Rojas & Brewer, 2008); Gómez et al., 2014) or cooked ground beef (J Ahn, Grün, & Mustapha, 2007; Rojas & Brewer, 2007) (J. Ahn, Grün, & Fernando, 2002). The antioxidant activity of GSE is concentration dependent between 0.02% and 0.1% (J. Ahn et al., 2002). (Rojas & Brewer, 2008) reported that the doses of GSE 0.01% and 0.02% were efficient in raw beef and pork patties. Furthermore, Gómez et al. (2014) showed that 250 mg GSE/kg meat inhibited the lipid oxidation in ground beef enriched with omega 3 and/or CLA. Likewise, the results of the present study indicated that GSE, with 95% of polyphenols, was effective against lipid oxidation, which would lead to a loss of sensory and nutritional properties, so the quality of beef patties could be improved by the addition of the dose of GSE used.

3.4. Colour

Table 6 shows the effects of beef, GSE and storage time on colour parameters (L*, a*, b*, C* and H*) of beef patties in aerobic packaging for 6 days under retail display conditions. The triple interaction BxGSExT was significant ($P<0.05$) for L*, b* and H*, whereas there was not interaction BxGSExT for a* and C* ($P>0.05$).

L* double interactions B×GSE was not significant during the display. There were no differences on L* values among the treatments according the beef used ($P>0.05$). L* values of beef patties were not affected by storage time ($P>0.05$), except the C-GSE0 treatment whose L* values slightly decreased over time ($P<0.05$). Gómez et al. (2014) reported that L* values at 0 day were similar than those of 6 day for ground beef. GSE addition had a significant effect on L* of beef patties at day 1 ($P<0.05$), resulting higher L* values in the GSE treatments. Gómez et al. (2014) observed no clear data trends by GSE addition in ground beef during 6 days of refrigerated storage.

The double interaction BxGSE was not statistically significant ($P>0.05$) for a* values over time. The a* values presented significant differences among treatments according to the beef used. Control patties showed lower a* values than those of other treatments at days 1 and 3. CLA addition resulted in a* values higher than those of Control treatments ($P<0.05$) until day 3 of refrigerated storage. Likewise, in previous

studies the enrichment of beef patties with CLA resulted a* values higher than beef without CLA ((Hur et al., 2004); Gómez et al., 2014). Beef patties enriched with omega 3 and/or CLA had higher a* values than those of Control treatments (P<0.05) at days 1 and 3; these results are agreement with those obtained by Gómez et al. (2014). All treatments showed a significant (P<0.001) decrease in redness (around 55%) because the pigments are oxidized during refrigeration of meat products (Faustman, Sun, Mancini, & Suman, 2010). GSE addition had a significant effect (P<0.05) on a* values of beef patties at day 1, resulting higher values for a* in the GSE treatments. These results support those obtained by Gómez et al. (2014) and (Rojas & Brewer, 2007) that did not find changes of redness in beef patties when GSE was added.

The b* significant interactions of BxGSE were not found over time except at 1 day. There were significant differences (P<0.05) on b* values according to the beef used at day 3, when control beef patties had higher b* values than those of the other beef patties enriched with omega 3 and/or CLA (9.96 *vs* 8.48, P<0.05). The yellowness was mainly affected by storage time (P<0.001) and the b* values decrease around 32% in the treatments. The b* values were influenced by the addition of GSE at 1 and 3 days (P<0.05), resulting lower values for b* in the GSE treatments at 3 day, whereas that GSE beef patties enriched with omega 3 and/or CLA had higher b* values than those of beef patties enriched and without GSE. However, Gómez et al. (2014) did not find differences when GSE was added to ground beef, but it can be explained because the olive oil used in the present study could have interacted with the GSE and affected to the yellowness.

The double interaction BxGSE was statistically significant (P<0.05) for C* values at day 1. The beef patties enriched with CLA had the highest C* values at day 3 (13.04 *vs* 11.46). There was no clear trend when GSE was added.

The H* significant interactions of BxGSE were found over time except at 0 day. There were significant differences (P<0.05) on H* values according to the beef used at days 1 and 3, when control beef patties had higher H* values than those of the other beef patties enriched with omega 3 and/or CLA. The H* values were influenced by the addition of GSE at 3 day (P<0.05), resulting lower values for H* in the GSE treatments.

There was a decrease in C* (around 45%, P<0.001) while H* increased (around 32%, P<0.05) during display, which are normally associated with the loss of redness

(Bañón et al., 2007) and the loss of stability of the colour in meat. In general, the addition of 250 mg GSE/kg meat did not affect the C* and H* values during display of beef patties in PVC packaging. These results are in agreement with those obtained by Gómez et al. (2014). In contrast, Bañón et al. (2007) found differences for C*and H* in beef patties with 100 SO_2+300 GSE (mg /kg meat) compared to patties without additives.

In the present study the samples were in aerobic packaged (PVC) which promoted the development of aerobic microorganisms and, consequently, resulting the discolouration of the beef. Lavieri & Williams (2014) used PVC packaging in ground beef and observed an increase of the bacterias psychrotrophic counts which led to discolouration in the ground beef for the refrigerated storage. Likewise, Gómez et al. (2014 data not published yet) observed the colour degradation of ground beef in PVC packaging during 6 days of refrigerated display. Therefore, in the present study it would be necessary to apply the hurdle technology by using natural antioxidant such as GSE, as well as additives and modified atmosphere or vacuum packaging, to protect fully these meat products from the discolouration and microbial growth.

The contradictory results with other authors can be explained due to the differences in methods to enrich the beef with omega-3 and CLA, grinding, salt, colour of GSE, different ratios lean/fat, light, composition of olive oil emulsion, storage time and packaging system used, which make difficult to compare the results. For instance, as result of omega-3 and CLA supplementation in diet compared to omega-3 and CLA directly added to meat, these fatty acids are located at different positions and some functions could be different. Likewise, the effect of the olive oil in emulsion can differ if it was added in encapsulated or directly. The grinding of meat accelerates oxidation of the pigments in the ground beef compared to the whole muscle (Honikel, 2004), whereas salt promotes lipid oxidation and accelerates the discolouration in meat (Rhee, 1999).

3.5. Sensory analysis

3.5.1. Raw ground beef sensory analysis

Evolution of colour and odour of the raw beef patties in aerobic packaging for 6 days under retail display conditions are shown in Fig. 1 and Fig. 2 respectively. There

was a significant interaction BxGSExT (P<0,001) for colour whereas for the odour it was not (P>0.05).

The double interaction BxGSE was significant for colour at days 3 and 6. In these days, the beef patties enriched with CLA had better scores of colour than the control treatments (6.57 *vs* 8.22, 8.58 *vs* 9.62), which could mean that CLA had a positive effect on the colour and would support the instrumental colour results (Table 6). The same positive effect of CLA was observed by Gómez et al. (2014) in ground beef with and without GSE. Moreover, the scores of colour in beef patties enriched with omega-3 (OME3 and OME3+CLA treatments) depended on the presence o absence of GSE over time, so, no clear trends were observed. Colour values of treatments increased gradually during storage from 2.45 to 9.18 (P<0.001), which would correspond to the discolouration of the beef due to the lipid oxidation and the oxidation of pigments. The GSE did not significantly influence on colour over time. GSE patties had lower colour scores than those of patties without GSE at day 6, although this trend (P=0.080) was not observed for patties enriched with omega-3 (OME treatments). Furthermore, it should be noted that GSE addition improved the colour value of OME3+CLA treatment at day 6. Gómez et al. (2014) did not clear differences for visual colour when 250 mg GSE/ kg meat was added to ground beef during 6 days of display. Likewise, 0.02% of GSE did not affect for visual colour of frozen beef (Rojas & Brewer, 2008).

The no significant interactions BxGSE were found for odour over time. Odour was not affected by beef during display, except at day 6 (Fig. 3), when CLA improved the odour values compared to those of other beef patties (7.60 *vs* 8.70, P<0.001). These results could mean that CLA had a positive effect on the odour at day 6. Moreover, omega-3 addition did not affected on odour values of beef patties during the 6 days, as well as Gómez et al. (2014) observed in ground beef. Odour values of treatments increased over time from 2.57 to 8.43 (P<0.001) by the secondary products of the lipid oxidation that happens during refrigerated storage (Jongberg, Skov, Tørngren, Skibsted, & Lund, 2011) and the spoilage of the beef due to microbial populations which lead to the formation of microbial slime formation, off-odour and discolouration (Lavieri & Williams, 2014). GSE addition affected odour scores resulting lower values in GSE beef patties from 1 day. However, Rojas & Brewer (2008) did not find differences for odour described as raw meat, grassy, herbal, acid and sweaty in beef.

In the present study, odour and colour deteriorated similarly over the time of display. The scores in both sensory parameters were higher than acceptability value (7.5 cm) from day 3, and the colour was the limiting parameter because the scores were higher those of odour scores at that day. In general, the shelf life would be established in 3 days for these raw beef patties packaged in PVC and stored under retail display conditions, results which support those obtained by Lavieri & Williams (2014) and Gómez et al. (2014). Furthermore, GSE addition improved the odour in Control and CLA treatments at day 6, by decreasing the odour values below the limit of acceptability.

3.5.2. Cooked ground beef sensory analysis

The Fig. 3 shows the colour and odour values of cooked beef patties at day 2. No significant interaction of BxGSE was found (P>0.05) for both sensory parameters. All treatments had colour and odour values lower than acceptability value (7.5cm), so all of them were sensorially acceptable.

There were no differences among types of beef for colour scores of beef patties (P>0.05). However, Gómez et al. (2014) found that the colour of cooked beef enriched with omega-3 or CLA were worse evaluated than control beef. Maybe, in the present study, the olive oil emulsion added has decreased the possible differences among the types of the beef. The colour scores improved when GSE was added to the beef patties (P=0.006). However, in previous studies GSE addition did not affect the colour of cooked ground beef during storage refrigerated (Gómez et al., 2014; Bañón et al., 2007; Jongberg et al., 2011). It should be noted that some compound of olive oil might have interacted with those of GSE during the cooking, by improving the colour scores of the beef patties.

Beef did not affect odour (P>0.05) of cooked beef patties. In previous study, Gómez et al. (2014) found that control treatments were the best evaluated compared to the enriched beef. These differences can be explained as the emulsion can have masked the possible differences among the types of the beef. The warmed over flavour (WOF) are usually developed in cooked beef within 1 to 3 days of refrigerated storage, but they were not detected in the beef patties at 2 day of the present study. There were no significant differences (P=0.209) when GSE was added to the beef patties of the present

study. Likewise, Bañón et al. (2007) did not find significant changes in the odour of cooked beef patties with GSE and low concentrations of sulfite. However, other studies found better scores in the odour parameter (Gómez et al., 2014) and the wet cardboard and rancidity parameters (Rojas & Brewer, 2007) when GSE was added to beef. The different results among the studies could be explained by the presence of additives and other ingredients, packaging type, lipid composition of beef and the content of polyphenolic compounds of GSE used in each of the studies.

In order to assess the acceptability of beef patties enriched with omega-3, CLA and oleic fatty acids, a hedonic sensorial test was carried out. Means scores given by the panellist for the beef patties are shown in Fig. 4 A-B.

No significant differences (P>0.05) were observed among types of beef for the parameters compared to the control beef patties. Jugosidad showed a slightly higher score for OME3 beef patties (Fig. 4.A), as well as the flavour característico provided significantly (P<0.05) higher values for OME3 patties compared to the others kinds of beef (8.03 *vs* 6.16). Moreover, panellist did not find significant differences (P>0.05) among groups for the overall acceptability, and the sensory panel ratings ranked the kinds of beef: OME3 > C = CLA > OME3+CLA. These results indicated that the enrichment of omega-3 in beef slightly improved the acceptability of beef patties. However, when the omega-3 is added combined with CLA, these light improvements were not observed. These findings would agree with those of Realini et al. (2014), who did not observed hedonic advantages in beef enriched with omega-3 plus CLA over conventional beef, whereas the individual beef enrichment with omega-3 or CLA improved consumer liking scores.

There were no significant differences for sensory parameters (*P*>0.05) when GSE was added to the beef patties of the present study (Fig. 4.B). GSE beef patties showed slightly higher jugosidad (6.47 *vs* 6.30) y slightly higher flavour característico (6.78 *vs* 6.48) than the beef patties without GSE. Furthermore, panellists did not find significant differences (P>0.05) between treatments with or without GSE for the overall acceptability, which was found to be GSE-250 > GSE-0 (8.45 *vs* 7.89). These results indicated that the addition of GSE as natural antioxidant slightly improved the acceptability of beef patties. This positive effect on the acceptability of meat products when GSE is added also was observed by Lorenzo et al. (2013).

3.6. Principal components analysis

The table 7 shows the correlation coefficients among the variables studied in the raw beef patties (TBARS, pH, L*, a*, b*, C*, H*, colour and odour). The colour coordinates a*, b* and C* were positively correlated to each other, and negatively correlated to H*. L* and pH were not correlated to neither variable. Moreover, colour and odour had high correlation coefficient to each other. Likewise, the correlation between a*, b* and C* to sensory parameters was negative and high, whereas H* was correlated positively and significantly to sensory parameters. TBARS was negatively correlated to a* and C*, and positively correlated to H*, colour and odour. These results would corroborate the relationship between lipid and myoglobin oxidation, which lead to off-flavour development and discolouration, respectively (Faustman et al., 2010).

The principal component analysis (PCA) showed that about 97.99% of variability was explained by four main principal components, and 70.52% of it was accounted for the principal component 1 (PC1). The increase in H*, colour and odour and the decrease in a*, b* and C* clearly reflect a degradation in beef patties quality; hence, PC1 was a beef patties quality degradation factor. The PC2 (12.49%) was correlated to variable TBARS, whereas the PC3 (9.47%) and the PC4 (5.51%) were formed by pH and L*, respectively.

When plotting the treatments of raw beef patties for 0 and 6 days on the same bi-dimensional space, a clear separation was observed by PC1 (Fig. 5). Treatments at day 0 were placed on the left side, whereas treatments at 6 day were placed on the right side. Consequently, the variables H*, colour and olour increased with increasing storage time, whereas a*, b* and C* decreased, so they can be used as indicators of the loss of raw beef patties quality. Likewise, Insausti et al. (2008) and Gómez et al. (2014) reported a clear separation by factor 1, related with the beef quality degradation, when plotting days of storage on the same bi-dimensional space.

Moreover at 6 day, PC2 separated GSE treatments (negative side of PC2) from the treatments without GSE (positive side of PC2). The PC2 was related with TBARS, so the lipid oxidation can be used as indicator of the effectiveness of GSE in raw beef patties at 6 day of display. The PCA also separated sausages with GSE from the control group (without antioxidants) by factor 1, which was positively related with moisture

content, aw, colour parameters and acetic concentration and inversely related with TBARS and TPA (Lorenzo et al., 2013). Gómez et al. (2014) differentiated the ground beef without added GSE according the enrichment or no with omega-3 and/or CLA, because CLA presented antioxidant effect in the ground beef. However, in the present study the treatments without GSE were not separated by PC2 at 6 day, because CLA did not have antioxidant effect in the patties without GSE. These differences between results it can be explained due to the olive oil addition, with high content of MUFA, which promoted the oxidation in the present study. So in the present study, the PC2 could not differentiate the beef patties according the enrichment or no with omega-3 and/or CLA.

4. Conclusions

The enrichment of beef with omega-3 and CLA improves the lipid profile of the beef, although the oxidative stability is impaired. The enrichment of omega-3 and omega-3 plus CLA by modifying the diet of bulls have not been enough to cause variations on the instrumental colour neither the sensory parameters, so, the visual appearance of enriched beef is similar to conventional beef. The results pointed to the potential value of CLA enrichment to stabilize the lipid oxidation. Furthermore, the colour in beef enriched with CLA was improved until day 3, which would be interesting to obtain more attractive products for the consumers. According to the sensory analyses, the shelf life of the ground enriched beef would be established in 3 days, under aerobic packaging and retail display conditions.

GSE addition prevented rancidity in raw beef patties enriched with unsaturated fatty acids and did not affect the instrumental colour neither the sensory parameters in ground beef. The results suggest that GSE can be a technologically viable alternative for stabilizing the lipid oxidation in new fresh meat products, although it should be used in conjunction the hurdle technologies to reduce the discolouration and the microbial growth.

Acknowledges

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Table 1. Fatty acid composition of raw beef.

Beef [a]	**C**	**OME3**	**CLA**	**OME3+CLA**
Fatty acid profile (% total fatty acid)				
Saturated Fatty Acid	42.51	37.84	39.68	38.98
Monounsaturated Fatty Acid	54.84	58.17	57.48	56.49
Polyunsaturated Fatty Acid	2.65	3.98	2.84	4.53
Fatty acid content (mg FA /100 g ground beef)				
Omega-3	1.46	5.87	1.70	6.39
CLA	9.16	7.78	12.56	11.20

[a] Beef from animals fed different diets: C, conventional; OME3, omega-3; CLA, conjugated linoleic acid; OME3+CLA, omega-3 plus CLA.

Table 2. Formulation (%, w/w) of different patties.

Treatments [a]	Beef	Olive oil emulsion			Salt	Grape seed extract
		Olive oil	Soy protein isolate	Water		
C–GSE-0	90.200	2.0	0.8	5.0	2.0	0.000
C–GSE-250	90.177	2.0	0.8	5.0	2.0	0.025
OME3–GSE-0	90.200	2.0	0.8	5.0	2.0	0.000
OME3–GSE-250	90.177	2.0	0.8	5.0	2.0	0.025
CLA–GSE-0	90.200	2.0	0.8	5.0	2.0	0.000
CLA–GSE-250	90.177	2.0	0.8	5.0	2.0	0.025
OME3+CLA–GSE-0	90.200	2.0	0.8	5.0	2.0	0.000
OME3+CLA–GSE-250	90.177	2.0	0.8	5.0	2.0	0.025

[a] Beef from animals fed different diets: C, conventional; OME3, omega-3; CLA, conjugated linoleic acid; OME3+CLA, omega-3 plus CLA. GSE used: GSE-0, without GSE; GSE-250, 250 mg GSE/kg meat.

Table 3. Proximate composition (means ± standard deviations) of polyunsaturated fatty acids enriched beef patties and with added grape seed extract and olive oil.

	C	OME3	CLA	OME3+CLA
Moisture (%)	68.54±2.026	69.80±0.263	69.42±0.738	69.85±0.102
Protein (%)	21.13±0.226	20.54±0.077	20.20±0.154	20.48±0.050
Fat (%)	6.91±1.880	6.79±0.675	6.78±0.639	6.87±0.356

[a] Beef from animals fed different diets: C, conventional; OME3, omega-3; CLA, conjugated linoleic acid; OME3+CLA, omega-3 plus CLA.

Table 4. Evolution of pH values in polyunsaturated fatty acids enriched beef patties and with added grape seed extract and olive oil stored in aerobic packaging for 0 and 6 days under retail display conditions.

GSE [a]	**GSE-0**				**GSE-250**					***P*-value**		
Beef [b]	**C**	**OME3**	**CLA**	**OME3+CLA**	**C**	**OME3**	**CLA**	**OME3+CLA**	**SEM**	**B**	**GSE**	**BxGSE**
Days												
0	5.56 [ab]	5.55 [ab]	5.76 [a]	5.50 [b]	5.56 [ab]	5.71 [ab]	5.52 [b]	5.57 [ab]	0.047	0.090	0.985	0.002
6	5.61	5.65	5.46	5.46	5.56	5.48	5.52	5.48	0.113	0.708	0.666	0.752
SEM	0.138	0.055	0.061	0.055	0.113	0.096	0.046	0.085				
P-value	0.797	0.260	0.015	0.561	0.976	0.131	0.942	0.505				

[a] GSE used: GSE-0, without GSE; GSE-250, 250 mg GSE/kg meat.
[b] Beef from animals fed different diets: C, conventional; OME3, omega-3; CLA, conjugated linoleic acid; OME3+CLA, omega-3 plus CLA. Mean values in the same row (different treatments on the same storage day) with different letter presented significant differences ($P<0.05$). SEM: Standard Error of Mean.

Table 5. Evolution of thiobarbituric acid reagent substances (TBARS, mg MDA/kg meat) in polyunsaturated fatty acids enriched beef patties and with added grape seed extract and olive oil stored in aerobic packaging for 0 and 6 days under retail display conditions.

GSE [a]	**GSE-0**				**GSE-250**					***P*-value**		
Beef [b]	**C**	**OME3**	**CLA**	**OME3+CLA**	**C**	**OME3**	**CLA**	**OME3+CLA**	**SEM**	**B**	**GSE**	**BxGSE**
Days												
0	0.44	0.67	0.39	0.44	0.38	0.50	0.32	0.42	0.065	0.011	0.105	0.668
6	5.22 [ab]	6.35 [a]	4.88 [b]	5.14 [b]	1.34 [c]	0.85 [c]	0.57 [c]	0.36 [c]	0.254	0.005	0.000	0.026
SEM	0.072	0.248	0.366	0.082	0.174	0.182	0.042	0.045				
P-value	<0.001	<0.001	<0.001	<0.001	0.008	0.221	0.006	0.386				

[a] GSE used: GSE-0, without GSE; GSE-250, 250 mg GSE/kg meat.
[b] Beef from animals fed different diets: C, conventional; OME3, omega-3; CLA, conjugated linoleic acid; OME3+CLA, omega-3 plus CLA. Mean values in the same row (different treatments on the same storage day) with different letter presented significant differences ($P<0.05$). SEM: Standard Error of Mean.

Table 6. Evolution of Lightness (L*), Redness (a*), Yellowness (b*), Chroma (C*) and Hue angle (H*) in polyunsaturated fatty acids enriched beef patties and with added grape seed extract and olive oil stored in aerobic packaging for 0, 1, 3 and 6 days under retail display conditions.

GSE [a]		**GSE-0**				**GSE-250**					***P*-value**		
Beef [b]		**C**	**OME3**	**CLA**	**OME3+ CLA**	**C**	**OME3**	**CLA**	**OME3+ CLA**	**SEM**	**B**	**GSE**	**BxGSE**
	Days												
L*	0	40.03 [1]	36.02	37.27	39.46	35.94	38.45	39.58	41.11	1.637	0.295	0.619	0.150
	1	36.60 [2]	34.59	35.91	36.50	38.28	37.21	38.36	39.55	1.147	0.309	0.003	0.945
	3	36.02 [2]	37.95	39.04	38.71	39.76	34.99	37.21	37.52	1.100	0.378	0.474	0.057
	6	37.52 [12]	39.84	40.55	40.88	38.17	37.78	39.89	38.93	1.482	0.309	0.928	0.162
	SEM	0.963	1.604	1.422	1.163	1.694	0.892	1.455	0.920				
	P-value	0,012	0.239	0.177	0.148	0.471	0.342	0.624	0.070				
a*	0	15.96 [1]	19.12 [1]	18.67 [1]	16.57 [1]	17.71 [1]	16.35 [1]	18.00 [1]	15.82 [1]	1.344	0.401	0.523	0.422
	1	11.99 [2]	11.83 [2]	13.85 [2]	11.76 [2]	11.51 [2]	14.40 [1]	14.25 [1]	13.70 [1]	0.660	0.009	0.020	0.090
	3	5.92 [3]	7.10 [2]	9.49 [3]	7.09 [3]	6.62 [3]	8.85 [2]	9.02 [2]	7.57 [2]	0.464	0.000	0.065	0.130
	6	7.49 [ab3]	9.19 [ab2]	7.66 [ab3]	7.30 [ab3]	6.42 [b3]	7.28 [ab2]	9.40 [a2]	7.73 [ab2]	0.641	0.080	0.659	0.037
	SEM	0.529	1.402	0.971	0.541	0.775	0.763	0.906	0.658				
	P-value	<0,001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001				
b*	0	12.30	14.83 [1]	13.88 [1]	13.65 [1]	12.88 [1]	12.09 [1]	13.30 [1]	12.80 [1]	1.202	0.844	0.293	0.581
	1	11.64 [a]	8.68 [bc2]	9.48 [abc2]	8.21 [c2]	9.06 [abc2]	10.34 [abc12]	11.35 [ab12]	11.34 [ab1]	0.624	0.399	0.024	<0.001
	3	10.06	8.75 [2]	9.71 [2]	9.42 [2]	9.85 [12]	7.16 [2]	8.52 [2]	7.33 [2]	0.534	0.002	0.001	0.352
	6	10.52	8.92 [2]	9.09 [2]	9.34 [2]	7.73 [2]	9.10 [12]	8.70 [2]	8.61 [2]	0.748	0.992	0.087	0.232
	SEM	0.712	1.145	0.900	0.556	0.808	0.829	0.736	0.628				
	P-value	0.147	0.001	0.003	<0.001	0.002	0.002	<0.001	<0.001				

GSE [a]		GSE-0				GSE-250					*P*-value		
Beef [b]		**C**	**OME3**	**CLA**	**OME3+ CLA**	**C**	**OME3**	**CLA**	**OME3+ CLA**	**SEM**	**B**	**GSE**	**BxGSE**
	Days												
C*	0	20.19 ^{1}	24.26 ^{1}	23.31 ^{1}	21.50 ^{1}	21.95 ^{1}	20.43 ^{1}	22.40 ^{1}	20.42 ^{1}	1.732	0.625	0.410	0.464
	1	16.75 ^{abc1}	14.79 ^{bc2}	16.83 ^{abc2}	14.38 ^{c2}	14.79 ^{bc2}	17.79 ^{abc1}	18.26 ^{a1}	17.83 ^{ab1}	0.774	0.119	0.008	0.003
	3	11.69 ^{2}	11.30 ^{2}	13.63 ^{2}	11.85 ^{2}	11.91 ^{23}	11.44 ^{2}	12.44 ^{2}	10.59 ^{2}	0.618	0.018	0.236	0.469
	6	12.97 ^{2}	12.85 ^{2}	11.89 ^{2}	11.86 ^{2}	10.07 ^{3}	11.68 ^{2}	12.95 ^{2}	11.60 ^{2}	0.851	0.679	0.183	0.149
	SEM	0.804	1.744	1.259	0.693	1.004	1.013	1.092	0.801				
	P-value	<0,001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001				
H*	0	37.17 ^{3}	37.69 ^{2}	36.77 ^{2}	39.16 ^{2}	35.92 ^{2}	35.77 ^{2}	36.36 ^{2}	38.98 ^{2}	1.364	0.190	0.332	0.916
	1	44.10 ^{a2}	37.08 ^{ab2}	34.28 ^{b2}	35.06 ^{b2}	37.49 ^{ab2}	35.37 ^{b2}	38.46 ^{ab12}	39.60 ^{ab2}	1.734	0.035	0.936	0.005
	3	59.41 ^{a1}	50.95 ^{bc1}	44.89 ^{cd1}	53.08 ^{ab1}	56.34 ^{ab1}	39.17 ^{d2}	43.45 ^{cd1}	43.99 ^{cd12}	1.699	<0.001	<0.001	0.009
	6	54.43 ^{a1}	43.15 ^{b2}	49.89 ^{ab1}	51.92 ^{ab1}	50.05 ^{ab1}	51.45 ^{ab1}	43.41 ^{b1}	48.33 ^{ab1}	2.305	0.075	0.353	0.013
	SEM	1.355	1.793	1.394	1.378	1.921	1.737	1.599	1.610				
	P-value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.011	0.006				

[a] GSE used: GSE-0, without GSE; GSE-250, 250 mg GSE/kg meat.
[b] Beef from animals fed different diets: C, conventional; OME3, omega-3; CLA, conjugated linoleic acid; OME3+CLA, omega-3 plus CLA.
Mean values in the same row (different treatments on the same storage day) with different letter presented significant differences (P<0.05).
Mean values in the same column (same treatment in different days) with different number presented significant differences (P<0.05).
SEM: Standard Error of Mean.

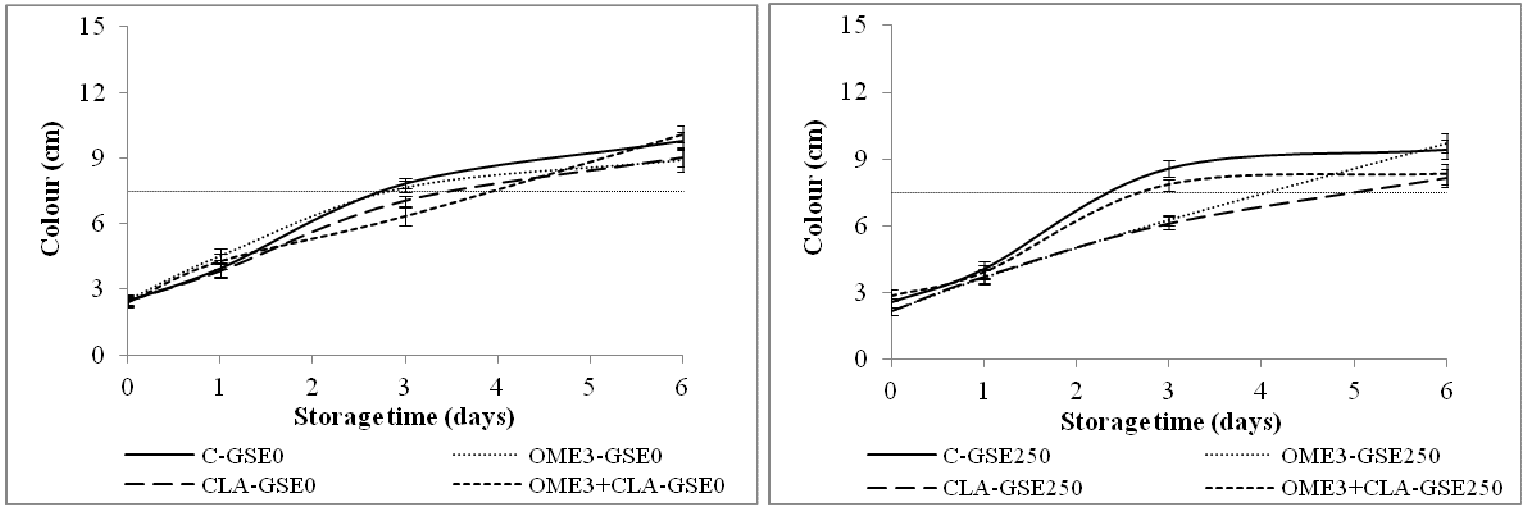

Fig. 1. Sensory evaluation: colour of the polyunsaturated fatty acids enriched beef patties and with added grape seed extract and olive oil, stored in aerobic packaging for 0, 1, 3 and 6 days under retail display conditions (15 cm: maximal decolouration scores; 7.5 cm: acceptability limit).
Beef from animals fed different diets: C, conventional; OME3, omega-3; CLA, conjugated linoleic acid; OME3+CLA, omega-3 plus CLA.
GSE used: GSE-0, without GSE; GSE-250, 250 mg GSE/kg meat.

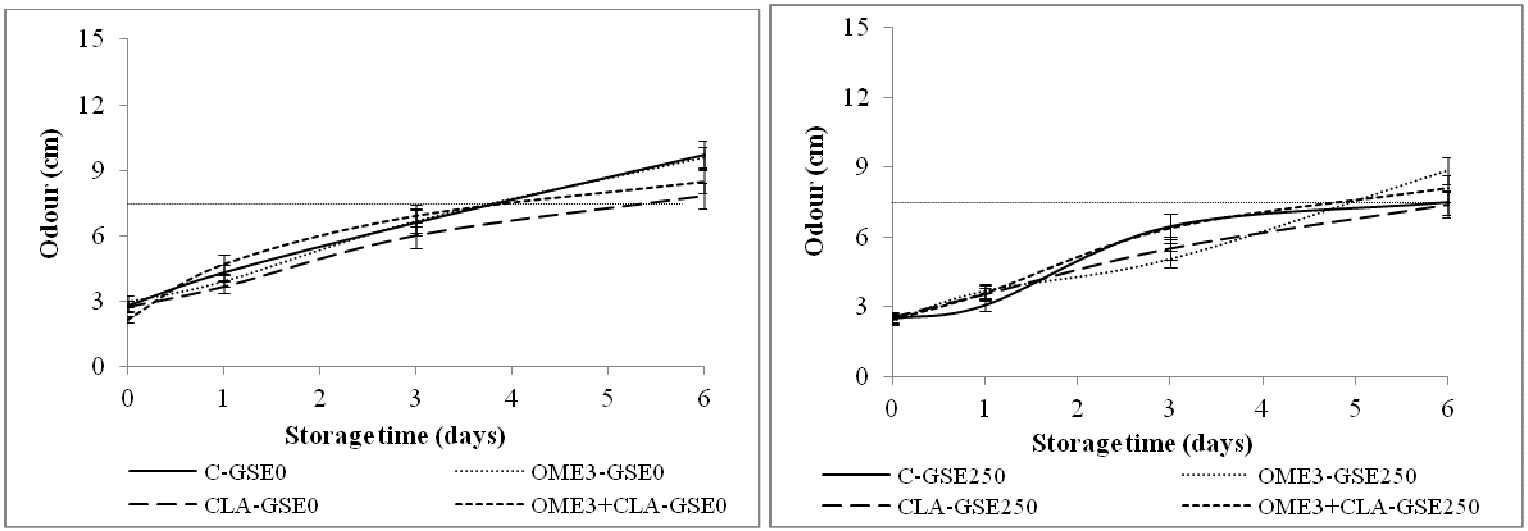

Fig. 2. Sensory evaluation: odour of the polyunsaturated fatty acids enriched beef patties and with added grape seed extract and olive oil, stored in aerobic packaging for 0, 1, 3 and 6 days under retail display conditions (15 cm: maximal odour scores; 7.5 cm: acceptability limit).
Beef from animals fed different diets: C, conventional; OME3, omega-3; CLA, conjugated linoleic acid; OME3+CLA, omega-3 plus CLA.
GSE used: GSE-0, without GSE; GSE-250, 250 mg GSE/kg meat.

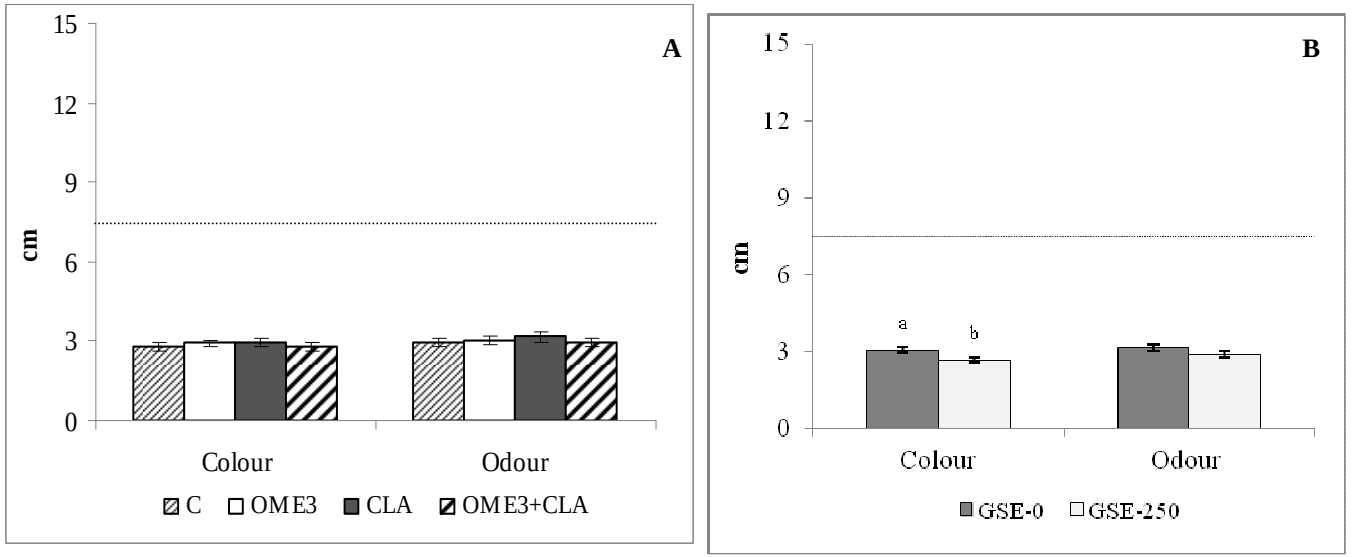

Fig. 3. Sensory evaluation of colour and odour (means ± SE) for the polyunsaturated fatty acids enriched beef patties and with added grape seed extract and olive oil, cooked at day 2 (15 cm: maximal decolouration and odour scores; 7.5 cm: acceptability limit). Effect of *beef* from animals fed different diets: C, conventional; OME3, omega-3; CLA, conjugated linoleic acid; OME3+CLA, omega-3 plus CLA (A). Effect of *GSE* used: GSE-0, without GSE; GSE-250, 250 mg GSE/kg meat (B).

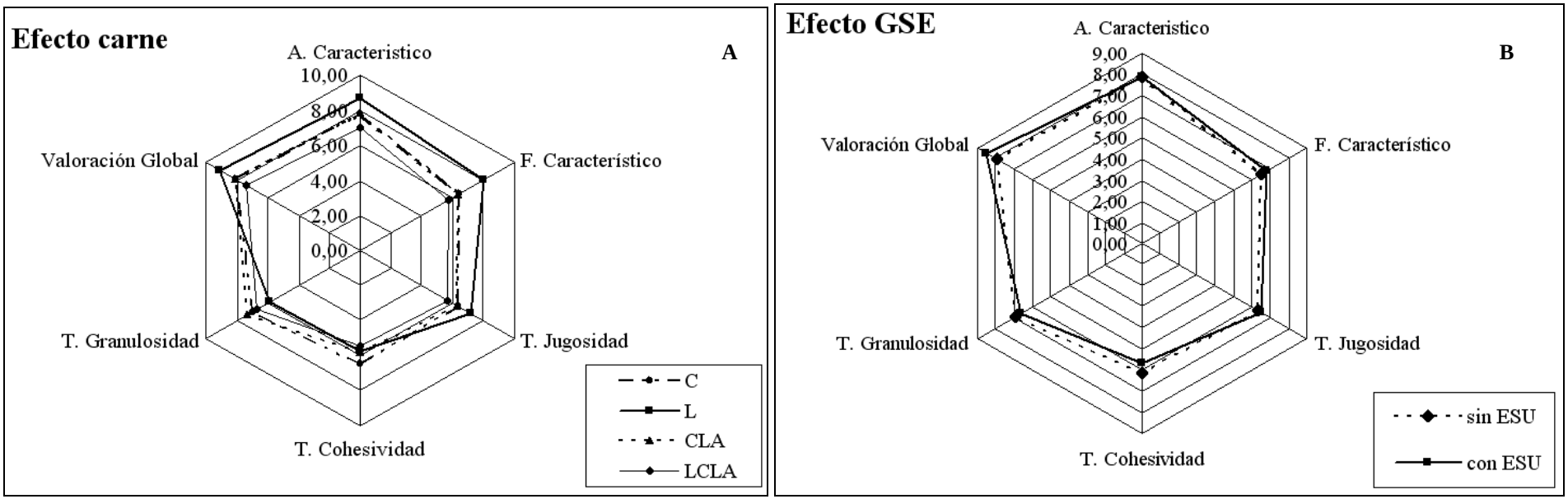

Fig. 4. Mean values of sensory properties of the polyunsaturated fatty acids enriched beef patties and with added grape seed extract and olive oil, cooked at day 2 (15 cm unstructured line scale: 0, sensation not perceived; 15, maximum of the sensation). Effect of *beef* from animals fed different diets: C, conventional; OME3, omega-3; CLA, conjugated linoleic acid; OME3+CLA, omega-3 plus CLA (A). Effect of *GSE* used: GSE-0, without GSE; GSE-250, 250 mg GSE/kg meat (B).

Table 7. Pearson's correlation coefficient among response variables: TBARS, pH, L*, a*, b*, C*, H*, colour and odour.

	TBARS	**pH**	**L***	**a***	**b***	**C***	**H***	**Odour**	**Colour**
TBARS	-								
pH	-0.056	-							
L*	0.289	-0.335	-						
a*	**-0.590***	0.413	-0.300	-					
b*	-0.478	0.347	-0.334	**0.953****	-				
C*	**-0.561***	0.401	-0.319	**0.995****	**0.978****	-			
H*	**0.602***	-0.447	0.165	**-0.935****	**-0.792****	**-0.898****	-		
Odour	**0.706****	-0.301	0.172	**-0.946****	**-0.877****	**-0.933****	**0.904****	-	
Colour	**0.661****	-0.381	0.213	**-0.978****	**-0.912****	**-0.967****	**0.942****	**0.981****	-

* Significance at level $P<0.05$.
** Significance at level $P<0.01$.

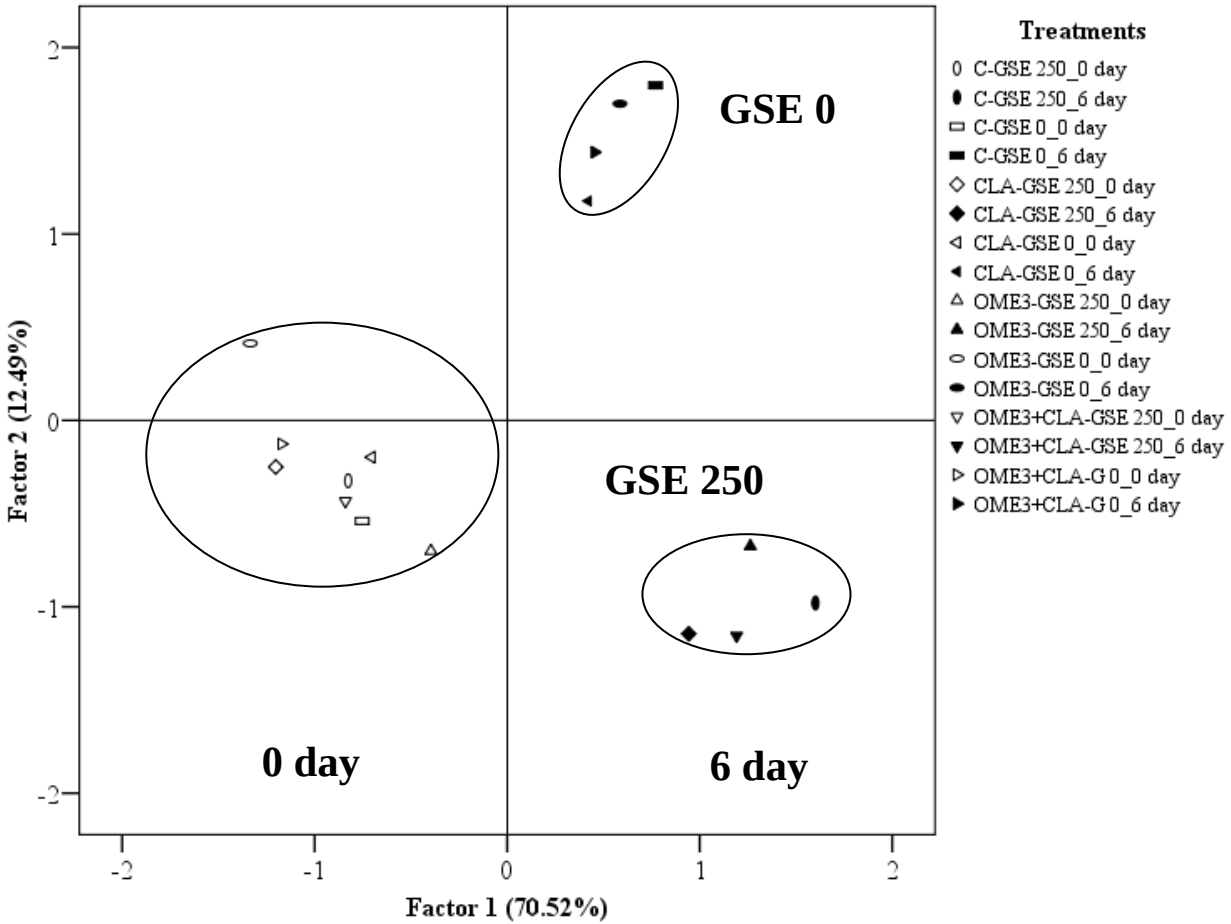

Fig. 5. Plot of treatments of raw beef patties for 0 and 6 days on the bi-dimensional space formed by factors 1 and 2 obtained by principal component analysis of TBARS, pH, L*, a*, b*, C*, H*, colour and odour variables.
GSE used: GSE-0, without GSE; GSE-250, 250 mg GSE/kg meat.
Beef from animals fed different diets: C, conventional; OME3, omega-3; CLA, conjugated linoleic acid; OME3+CLA, omega-3 plus CLA.

7. Discusión general

En la presente Tesis Doctoral se ha estudiado el efecto de la incorporación de ingredientes ricos en ácidos grasos poliinsaturados n-3 y CLA en las dietas de cebo de terneros Holstein con objeto de mejorar la calidad nutricional y organoléptica y lograr una mayor competitividad dentro del sector cárnico. Para ello se han evaluado diferentes parámetros en la cadena de carne de vacuno, es decir, desde la cría del animal, en especial, estudiando el desarrollo y metabolismo de la grasa subcutánea e intramuscular, la evaluación de la calidad de la carne y la aceptabilidad por parte de los consumidores, hasta el desarrollo de nuevos derivados cárnicos.

La adición de semilla de lino, fuente natural de ácido linolénico, en la dieta de los terneros ha demostrado ser efectiva en el aumento de los ácidos grasos *n-3* en la carne de vacuno. La cubierta de esta semilla puede proporcionar protección a los PUFA frente a la biohidrogenación ruminal y así aumentar el paso de los PUFA al duodeno como indicaron Scollan *et al.* (2001). Por otra parte, la adición directa de CLA debe ser en forma protegida, para prevenir su biohidrogenación en el rumen (Perfield *et al.*, 2004). Aunque ha habido experiencias previas con la utilización por separado de ambas materias primas, hasta el momento se desconocía el efecto que la adición conjunta de estos dos ingredientes pudiera tener sobre los parámetros productivos de los terneros y la calidad de la carne de vacuno.

La inclusión de un 10% de semilla de lino y un 2% de CLA en la dieta de los terneros no dio lugar a diferencias significativas en los parámetros de crecimiento, la canal y la composición tisular de la décima costilla. La ganancia media diaria de los animales fue bastante alta en contraste con la de otros estudios que estudiaron el efecto de la inclusión de semilla de lino en la dieta de terneros Holstein sobre la calidad de la carne (Mach *et al.*, 2006; Corazzin *et al.*, 2012). Los terneros Holstein tuvieron un rendimiento a la canal bastante similar al de otros Holsteins alimentados con dietas y sacrificado con pesos similares (Mach *et al.*, 2006). Además, estos terneros presentaron un alto porcentaje de hueso (22,3±0,76%) y un bajo porcentaje de músculo (58,4±0,96%), valores que corresponden a animales de raza Holstein, cuya selección está orientada hacia la producción de leche y, por tanto, presentan una conformación poco carnicera. Respecto al número y tamaño de adipocitos y la actividad de las enzimas lipogénicas estudiadas, no se ha encontrado un efecto significativo de la dieta suministrada a los terneros. Sin embargo, sí se encontraron

diferencias significativas entre depósitos grasos. El depósito intramuscular mostró un gran número de pequeños adipocitos que estarían indicando la presencia de una intensa hiperplasia de los adipocitos en dicho depósito. Si tenemos en cuenta que la hiperplasia de los adipocitos ocurre en un proceso temprano en el desarrollo de los depósitos grasos, mientras que la hipertrofia de los adipocitos es el proceso predominante en el desarrollo posterior (Robelin, 1986), se confirmaría que el depósito subcutáneo estaría en un estado más avanzado de desarrollo que el intramuscular.

La inclusión de un 10% de semilla de lino en las dietas L y L+CLA aumentó hasta 10 veces la proporción de ALA en los tejidos intramuscular y subcutáneo de los terneros del presente estudio. Asimismo duplicaron las proporciones de los ácidos grasos EPA y DPA, coincidiendo con resultados de estudios previos donde se informaron aumentos en los ácidos grasos EPA y DPA cuando las dietas eran enriquecidas en ALA (Noci *et al.*, 2007; Nassu *et al.*, 2011; Mapiye, Aalhus *et al.*, 2013). Aunque son considerables los aumentos logrados en el contenido en estos ácidos grasos *n-3*, la contribución de una ración de 200 g de estas carnes enriquecidas son pequeñas respecto a los consumos recomendados de ALA (25,78 mg *vs* 1,6 g/d para hombres y 1,1 g/d para mujeres; Institute of Medicine of the National Academies, 2002) y EPA más DHA (6,29 mg vs 250 mg, Musa-Veloso *et al.*, 2011).

Las proporciones del ácido graso ruménico se duplicaron en las carnes de los terneros alimentados con la dieta enriquecida con un 10% de semilla de lino. Esto podría estar relacionado con la posible biohidrogenación de parte de los ácidos grasos ALA y LA adicionados en la dieta que, mediante la biohidrogenación en el rumen por las bacterias *Butyrivibrio fibrosolvens*, genera ruménico que posteriormente es depositado en los depósitos grasos (Kepler *et al.*, 1966). En estudios previos, el aumento de CLA ha estado relacionado con el alto contenido de ALA en la dieta (Enser *et al.*, 1999; Stasiniewicz *et al.*, 2000; Raes *et al.*, 2003). Por otra parte, la adición de un 2% de CLA protegido en la dieta de los terneros dio lugar a aumentos en el contenido del ácido graso ruménico. Este aumento fue mayor cuando se adicionaron la semilla de lino y el CLA de manera conjunta, manifestándose un efecto aditivo.

Un valor aproximado de 4 en el ratio *n6-n3* es el recomendado desde el punto de vista nutricional (WHO, 2003). El ratio *n6-n3* descendió ligeramente en la carne de los terneros alimentados con las dietas enriquecidas con un 2% de CLA, pero sólo alcazaron los valores recomendados las carnes de los terneros alimentados con las dietas enriquecidas en lino, debido a los aumentos conseguidos en los ácidos grasos *n-3*.

Tras el análisis del perfil lipídico de las carnes de los terneros del presente estudio, se podría recomendar la dieta enriquecida con un 10% de lino y 2% de CLA para obtener carnes con un mejor perfil nutricional. Asimismo, desde el punto de vista sensorial, la carne de los animales alimentados con las dietas enriquecidas con semilla de lino mejoró aspectos de calidad como la terneza, la jugosidad, incluso a tiempos de maduración cortos (7 días). Por otro lado, la adición de CLA en las dietas de los terneros dio lugar a una reducción de la jugosidad y no afectó a la terneza (Barahona *et al.*, 2014). Respecto al estudio de consumidores realizado en las tres ciudades españolas (Barcelona, Zaragoza y Pamplona), la adición de semilla de lino o CLA en la dieta de los terneros mejoró la aceptabilidad de la carne. Además, hay que tener en cuenta que el empleo de estos ingredientes supone un coste añadido en el precio final de estas carnes enriquecidas, pero que los consumidores estarían dispuestos a pagar, tal y como revelan los resultados del estudio de consumidores. Por todo ello, el empleo de semilla de lino y CLA en los piensos tradicionales puede suponer una alternativa que mejore tanto la calidad nutricional como organoléptica de la carne de vacuno.

Aprovechar las partes menos comerciales de los terneros y darles un valor agregado, representa una oportunidad de negocio importante. Un alto grado de poli-insaturación acelera los procesos oxidativos dando lugar al deterioro en la carne del flavor, color, textura y valor nutricional (Mielnik *et al.*, 2006), limitando así la vida útil de estos productos. Además, operaciones de procesado como el picado o adición de aditivos como la sal promueven los procesos oxidativos. Por ello, el uso de antioxidantes resulta necesario. El extracto de semilla de uva (ESU) contiene sustancias polifenólicas y ha presentado actividad antioxidante en carne de vacuno cruda y cocinada (Ahn *et al.*, 2002; Bañón *et al.*, 2007; Rojas and Brewer, 2007, 2008; Schevey *et al.*, 2013). En el estudio de vida útil de las carnes de la presente

experiencia, la dosis de 250 mg ESU/kg carne fue suficiente para inhibir la oxidación lipídica. Cabe destacar el efecto antioxidante del CLA en la carne que, tras 6 días de exposición, presentó valores por debajo de los límites de rancidez establecidos para la aceptabilidad de los consumidores de carne de vacuno (2 mg MDA/kg; Campo *et al.*, 2006). Este efecto antioxidante del CLA coincide con el observado en trabajos previos de carne (Joo *et al.*, 2002; Chae *et al.*, 2004; Hur *et al.*, 2004). Asimismo, la carne enriquecida en CLA mostró una tendencia a mejorar las notas de valoración sensorial respecto al resto de carnes.

Una vez conocida la dosis apropiada para controlar los procesos oxidativos en las carnes enriquecidas en *n-3*, se realizó una segunda experiencia adicionando aceite de oliva a las hamburguesas, dado que la palatabilidad aumenta al aumentar la grasa hasta el 7% (Savell y Cross, 1988). Entre las grasas empleadas en los derivados cárnicos, el aceite de oliva es el lípido vegetal que ha recibido mayor atención porque es un aceite insaturado rico en antioxidantes naturales y una excelente fuente de ácidos grasos poliinsaturados (Jiménez *et al.*, 2010; Fernández *et al.*, 2009). Por tanto, se empleó el aceite de oliva en forma emulsionada y se estudió la vida útil de los productos. Nuevamente se volvió a mostrar la tendencia de mejores notas de valoración sensorial en las carnes enriquecidas en CLA. En general, las carnes enriquecidas con *n-3* y CLA no presentaron grandes diferencias respecto a la convencional en los parámetros estudiados, presentando una aptitud tecnológica adecuada para el desarrollo de nuevos derivados cárnicos.

Diversos equipos de investigación europeos (proyecto ProSafeBeef, coordinado por Teagasc) y españoles (INIA, RTA2009-00004-CO2, integrado por CITA, UNIZAR, IRTA y UPNA), trabajan con el objeto de que la cadena de carne de vacuno opere a niveles competitivos y sostenibles, ofreciendo productos seguros y de alta calidad a sus consumidores. En esta línea de mejora y avance en el sector, la presente Memoria de Tesis Doctoral ha desarrollado y estudiado una carne de vacuno enriquecida en ácidos grasos *n-3* y CLA mediante el empleo de materias primas ricas en ácidos grasos poiinsaturados en las dietas de los terneros. El enriquecimiento con un 10% de semilla de lino y 2% de CLA de las dietas, que no ha afectado a los parámetros productivos ni metabolismo del tejido adiposo, ha mejorado el perfil de ácidos grasos de la carne, adecuándose más a los requerimientos nutricionales de los

consumidores. Asimismo, esta carne enriquecida ha mejorado su calidad organoléptica en función de las preferencias de los consumidores y ha presentado una buena aptitud tecnológica. Finalmente, se considera que los resultados obtenidos pueden ayudar al sector del vacuno a competir más eficientemente dentro del sector cárnico.

8. Conclusiones

CONCLUSIONES

Con el material y métodos empleados y a partir de los resultados obtenidos, se ha llegado a las siguientes conclusiones:

1. La inclusión de un 10% de semilla de lino y/o un 2% de CLA protegido en dietas isoenergéticas e isoproteicas de terneros de raza Holstein durante su cebo no ha influido en los parámetros productivos, la calidad de la canal o el desarrollo y metabolismo del tejido adiposo. Por tanto, ambos ingredientes pueden utilizarse en esas proporciones en la alimentación de terneros sin que ello suponga una disminución de sus rendimientos productivos.

2. La actividad de las enzimas lipogénicas estudiadas, así como el número y tamaño de los adipocitos ha variado significativamente entre los depósitos grasos subcutáneo e intramuscular. Ello se ha debido a la diferente precocidad de ambos depósitos, el intramuscular más tardío que el subcutáneo, no habiendo un efecto significativo de la dieta suministrada a los terneros.

3. La modificación de las dietas de los terneros ha modificado el perfil lipídico de las carnes obtenidas. Mientras que la adición de un 2% de CLA protegido ha aumentado el contenido del ácido graso ruménico y ha descendido ligeramente el ratio n6-n3, la inclusión de un 10% se semilla de lino ha aumentado el contenido de los ácidos grasos ALA, EPA y RA y descendido significativamente el ratio n6-n3.

4. La adición conjunta de un 10% de lino y 2% de CLA ha mostrado un efecto aditivo en el aumento del ácido graso ruménico en la carne. Esta combinación es la que ha presentado un mejor perfil lipídico desde el punto de vista nutricional, debido al aumento en el contenido de los ácidos grasos n-3 de interés nutricional

(ALA, EPA y RA) y al descenso del ratio n6-n3, alcanzando las recomendaciones nutricionales de la EFSA.

5. El perfil lipídico tuvo tendencias similares en los depósitos intramuscular y subcutáneo dependiendo de la dieta suministrada a los terneros. A pesar de que el depósito intramuscular presentó mayores proporciones en ΣPUFA Σn-6 y Σn-3 y menores proporciones de ΣCLA, ΣMUFA y ΣSFA que el subcutáneo, ambos podrían ser empleados en la elaboración de derivados cárnicos enriquecidos en ácidos grasos n-3 y CLA.

6. La inclusión de semilla de lino o CLA en el cebo de terneros Holstein ha ofrecido ventajas sensoriales sobre la carne de animales alimentados con dietas no enriquecidas, mejorando la aceptabilidad de la carne por parte de los consumidores. Sin embargo, la carne enriquecida con n-3 y CLA no ha ofrecido ventajas hedónicas respecto a la carne convencional.

7. El factor con mayor influencia que condiciona a la mayoría de las decisiones de compra de los consumidores de carne en las tres ciudades estudiadas (Barcelona, Zaragoza y Pamplona) ha sido el contenido de grasa visible de la carne, prefiriendo las de poco veteado, seguido por su precio, origen y color. Por tanto, mediante la inclusión de un 10% de semilla de lino y un 2% de CLA en la dieta de los terneros se mejora la calidad nutricional de la grasa sin variar su contenido de grasa visible, lo que estaría acorde con la tendencia de los consumidores hacia el consumo de carnes magras.

8. Los consumidores del estudio han afirmado que estarían dispuestos a pagar un sobrecoste del 9, 11 y 15% por las carnes enriquecidas en CLA, *n-3* y *n-3* más CLA, respectivamente. Por tanto, se abren buenas expectativas para este tipo de carne enriquecida de ternera y, en consecuencia, para el desarrollo de nuevas

pautas productivas que posibiliten la obtención de carnes novedosas y atractivas para el consumidor.

9. El extracto de semilla de uva (ESU), en la dosis empleada (250 mg ESU/kg carne), ha inhibido la oxidación lipídica sin afectar al color ni los parámetros sensoriales de los derivados cárnicos elaborados con la carnes enriquecidas en ácidos grasos n-3 y/o CLA (carne picada y hamburguesas con aceite de oliva). Así, el ESU ofrece una solución tecnológica a los problemas de oxidación que aparecen en este tipo de productos.

10. La vida útil de las hamburguesas elaboradas con estas carnes enriquecidas y envasadas aeróbicamente se ha determinado que es de 3 días. Por ello, es necesario el uso de tecnologías barreras para prolongar la vida útil de este tipo de derivados cárnicos.

11. Dentro de los productos elaborados con carne picada y adición de sal, destacan los obtenidos con la carne enriquecida en CLA, que presentaron los menores niveles de oxidación lipídica respecto al resto de tratamientos de carne picada. Esto demuestra el efecto antioxidante del CLA en la carne cuando es adicionado en la dieta de los terneros.

9. Conclusions

CONCLUSIONS

Taking the results from this work into account, the following conclusions were reached:

1. Including 10% flaxseed and / or 2% CLA protected isoenergetic diets and isoproteic calf Holstein for your bait has not influenced the growth performance, carcass quality or development and adipose tissue metabolism. Therefore, both ingredients can be used in these proportions in feed for calves without entailing a decrease in growth performance.

2. Activity lipogenic enzymes studied, and the number and size of adipocytes has varied significantly between intramuscular and subcutaneous fatty deposits. This is due to the different earliness of both deposits, the intramuscular than subcutaneous later, not having a significant effect of the diet fed to calves.

3. The change in diets of calves has modified the lipid profile of meat produced. While the addition of 2% CLA protected increased content of rumenic fatty acid fell slightly N6-n3 ratio, including a 10% linseed has increased content of fatty acids ALA, EPA and RA and significantly decreased the ratio n6-n3.

4. The joint addition of a 10% flax and 2% CLA showed an additive effect on increasing the fatty acid in the meat rumen. This combination is what has produced a better lipid profile from the nutritional point of view, due to the increase in the content of fatty acids n-3 nutritional interest (ALA, EPA and RA) and declining ratio n6-n3, reaching the nutritional recommendations of the EFSA.

5. The lipid profile was similar trends in the intramuscular and subcutaneous deposits depending on the diet fed to calves. Although the intramuscular depot had higher proportions ΣPUFA Σn3 and Σn-6-3 and lower proportions of ΣCLA, ΣMUFA and subcutaneous ΣSFA that both could be used in the preparation of meat products enriched in n-3 fatty acids and CLA.

6. Including flaxseed or CLA in Holstein calves bait offered sensory advantages over meat fed diets not enriched animals, improving the acceptability of meat by consumers. However, the meat enriched with n-3 and CLA has offered hedonic advantages over conventional meat.

7. Factor with the greatest influence that determines most of the purchasing decisions of consumers of meat in the three studied cities (Barcelona, Zaragoza and Pamplona) is content visible fat from meat, preferring low marbled followed by price, origin and color. Therefore, by including 10% flaxseed and 2% of CLA in the diet of calves nutritional quality without varying fat content of fat is enhanced visible, which would be consistent with the tendency of consumers towards consumption of lean meats.

8. Consumers have stated that the study would be willing to pay an extra cost of 9, 11 and 15% meat enriched in CLA, n-3 and n-3 more CLA, respectively. Thus, great expectations for these veal meat enriched and consequently to the development of new production patterns that allow obtaining new and attractive for the consumer meat is open.

9. The grape seed extract (GSE), at the dose used (250 mg ESU / kg meat), inhibited the lipid peroxidation without affecting the color and sensory parameters of meat derived processed meat enriched with fatty acids n-3 and / or

CLA (minced meat and burgers with olive oil). Thus, ESU offers a technological solution to the corrosion problems that appear in this product.

10. The shelf life of these burgers made with meat packed aerobically enriched and has determined that it is 3 days. Therefore, the use of barriers technologies prolong the shelf life of such meat products is necessary.

11. Among the products made from minced meat and salt addition, include those obtained with meat enriched in CLA, which had the lowest levels of lipid oxidation compared to other treatments beef. This shows the antioxidant effect of CLA in meat when it is added to the diet of calves.

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